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The Role of Alpha Synuclein in Parkinson's disease

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The Role of Alpha Synuclein in Parkinson's disease

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Thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. It is characterized by the presence of intracellular inclusions termed Lewy bodies (LBs) and Lewy neuritis (LNs) in the brain, in which α -Syn aggregates constitute the main component. Therefore, α -Syn aggregation was implicated in the pathogenesis of PD. Structurally α -Syn is a disordered protein with little ordered structure under physiological conditions. However, research of α -Syn has provided substantial information about its structural properties. The precise function of α -Syn is still under investigation. Research has also shown that metals, such as copper and iron, accelerate α -Syn aggregation and fibrillation *in vitro* and are proposed to play an important role *in vitro*. In this study, isothermal titration calorimetry was used to determine iron binding properties to α -Syn revealing the presence of two binding sites for iron with an affinity of $1.06 \times 10^5 \text{ M}^{-1}$ and a dissociation constant of $\sim 10 \mu\text{M}$ which is physiologically relevant to iron content in the brain. In addition, α -Syn was found to reduce iron in the presence of copper. This property was demonstrated via ferrozine based assay. *In vitro*, thoflavin-T fluorescence assay was used to investigate the mechanism by which metals induce α -Syn aggregation and whether it is related to metal binding. Metals, mainly copper and iron, caused 2-fold increase in the aggregation rate of WT α -Syn and its metal binding mutants. Linking that to the increased metal content in the brain, α -Syn aggregation can cause changes in tissue composition, thus altering the normal functional environment in the brain. Moreover, western blotting analysis showed that copper increases the aggregate formation in mammalian dopaminergic cells over-expressing α -Syn.

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Publications

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Wang, X., Moualla, D., Wright, JA and Brown DR. (2010) Copper Binding Regulates Intracellular Alpha-Synuclein Localisation, Aggregation and Toxicity. *J. Neurochem.* 2010 May; 113(3): 704-14.

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Abbreviations

AD	Alzheimer's disease
AEC	Anion Exchange Chromatography
Ach	Acetylcholine
α -Syn	Alpha Synuclein
β -Syn	Beta Synuclein
BSA	Bovine Serum Albumin
CA	Catecholamines
CD	Circular Dichroism Spectroscopy
CEC	Cation Exchange Chromatography
DA	Dopamine
DLB	Dementia with Lewy Body Disease
DLBD	Diffuse Lewy Body Disease
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOPA	Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
FBS	Fetal Bovine Serum
FPLC	Fast Protein Liquid Chromatography
γ -Syn	Gamma Synuclein
IEC	Ion Exchange Chromatography
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl β -D-thiogalactopyranoside
IRPs	Iron Regulatory Proteins
ITC	Isothermal Titration Calorimetry
LBs	Lewy Bodies
LN	Lewy Neurites
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRE	Metal Response Element
MSA	Multiple System Atrophy
NAC	Non-A β -component of Alzheimer's disease Amyloid
NET	Norepinephrine Transporters
NMR	Nuclear Magnetic Resonance
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PK	Proteinase K
PLD2	Phospholipase D2
PMSF	Phenylmethanesulphonyl Fluoride
PVDF	Polyvinylidene Fluoride
ROS	Reactive Oxygen Species
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SNpc	Substantia nigra pars compacta
TESS	Transcriptional Element Software Search
THEMED	Tetramethylethylenediamine
TH	Tyrosine Hydroxylase
WT	Wild Type

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Chapter One: Introduction

1.1 Background of Parkinson's Disease

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. It has been defined as a movement illness accompanied by neuronal loss of dopaminergic neurons in the nigrostriatal system, in particular neurons in the substantia nigra pars compacta (SNpc) (Forno 1996; Dauer and Przedborski 2003). Parkinson's disease is classified as non-genetic disease, since the majority of cases occur sporadically and for unknown reasons. Epidemiological studies suggest that environmental factors may play an important role in most sporadic cases of the disease (Tanner 2003; Wirdefeldt, Adami et al. 2011) where no apparent genetic linkage was established (Dauer and Przedborski 2003). In concept, cases of sporadic appearance (ie, not inherited) is referred to as idiopathic PD (IPD). Nevertheless, some of the late onset cases of sporadic PD have been shown to result from and associate with genetic changes (Gilks, Abou-Sleiman et al. 2005) or a complex interaction between genetic and environmental factors (Maraganore, Farrer et al. 2003).

The aetiology of PD is not well known and regardless of the abundance of sporadic cases of the disease, approximately 10% of the cases are thought to have genetic origin. The identification of inherited forms of PD is considered to be a crucial step forward in understanding the mechanisms behind the disease pathology. Mendelian inheritance of the disease trait or the presence of the disease among patients with familial relations introduced the term, familial PD. Several gene mutations have been described in patients with familial forms of the disease, including mutations in alpha synuclein gene and Parkin gene (Hardy, Cai et al. 2006).

Specific clinical criteria has been established for IPD based on late age of disease onset, typically in the seventh decade, the absence of a family history and is confirmed by a post-mortem analysis showing neuronal loss in the brain and the presence of Lewy bodies (LBs) and Lewy neuritis (LNs) in the surviving neurons (Figure 1.1). The presence of the classical symptom triad of the disease, such as resting tremor, rigidity, bradykinesia in addition to good response to levodopa therapy (Gibb 1988; Gibb and Lees 1988) are also included in the clinical criteria of PD. Both familial and idiopathic PD is almost clinically indistinguishable. Nevertheless, patients with familial disease can display some features that are not found in patients with IPD, such as early age of disease onset which ranges between 30-60 years (Olanow and McNaught 2006).

Fig. 1.1 α -Syn-positive inclusions in patients with neurodegenerative disease. Inclusions in patients with Dementia with Lewy body (DLB) (a and b), and with PD (c) immunostained for alpha synuclein. a, α -Syn-positive Lewy neurites in the SNpc. b, α -Syn-positive Lewy body (arrow) in pigmented nerve cell of the SN (Spillantini, Schmidt et al. 1997). c, Lewy bodies in the SNpc of PD brain.

1.2 Familial Parkinson's disease

Several gene mutations have been described in patients with familial form of the disease (Hardy, Cai et al. 2006).

1.2.1 Alpha-synuclein gene

Alpha-synuclein gene (SNCA) was the first gene to be associated with familial PD. Three point mutations, as well as duplications and triplications in the SNCA gene, have been described in affected individuals with the disease. The first point mutation, causing alanine to threonine substitution at position 53 of the alpha-synuclein (α -Syn) protein (Ala53Thr), has been first reported in Italian-American family known as the Contursi kindred and in another three unrelated families of Greek origin (Polymeropoulos, Lavedan et al. 1997). This was followed by the recognition of another form of α -Syn mediated familial PD within patients of German origin with a second point mutation resulting in the substitution of alanine to proline at residue 30 (Ala30Pro) (Kruger, Kuhn et al. 1998). More recently, a third point mutation in SNCA gene causing the substitution of glutamate with lysine at residue 46 (Glu46Lys) was discovered in a Spanish kindred (Zarranz, Alegre et al. 2004). PD patients carrying the Ala30Pro mutation display symptoms that clinically overlap with IPD (Kruger, Kuhn et al. 2001). In contrast, carriers of the Ala53Thre mutation have a more rapid disease progression with an early age of disease onset (Schiesling, Kieper et al. 2008), while carriers of the Glu46Lys mutations have a higher occurrence of dementia (Zarranz, Alegre et al. 2004). Patients with duplications and triplications in SNCA are clinically similar to IPD patients, however, there is increasing severity with increasing gene multiplication, for example more rapid disease progression and early dementia in carriers of the gene triplications (Fuchs, Nilsson et al. 2007). Moreover, a polymorphism in the promoter region of SNCA has been identified as a risk factor for the disease in patients of different ethnic origins (Maraganore, de Andrade et al. 2006). These observations along with the discovery of full length α -Syn protein in LBs from post-mortem brain samples of patients with sporadic PD as well as familial PD (Spillantini, Schmidt et al. 1997; Wakabayashi, Matsumoto et al. 1997; Baba, Nakajo et al. 1998) strongly suggest that α -Syn plays an important role in the pathogenesis of Parkinson's disease.

1.2.2 Other forms of familial Parkinson's disease

Genes other than SNCA have been linked and classified as potential causes or risk factors in familial PD. Mutations in the Parkin gene (PARK2) have been identified in Japanese families, as well as in patients of different ethnic origins, as the likely cause of early-onset Parkinsonism (Klein, Lohmann-Hedrich et al. 2007). Patients with mutations in the Parkin gene share similar clinical symptoms with IPD patients, but have earlier age of onset and slower disease progression (Lohmann, Periquet et al. 2003). The ubiquitin C-terminal hydrolase-1 (UCH-L1) gene (PARK5), coding for the multifunctional ubiquitin C-terminal hydrolase-1, has been classified as the third gene to be associated with familial PD in a German family. Patients with the mutation in the PARK5 gene, although rare, display the clinical features of IPD (Leroy, Boyer et al. 1998). Other studies have shown that modifications of the peptide sequence of UCH-L1, due to oxidative damage, are linked to neurodegeneration found in brains of patients with Alzheimer's disease (AD) and PD (Castegna, Aksenov et al. 2002; Choi, Levey et al. 2004). In addition to Parkin (PARK2) and UCH-L1 (PARK5) genes, familial PD has also been linked to mutations in other genes such as the PINK1 gene (PARK6) (Valente, Brancati et al. 2002; Valente, Abou-Sleiman et al. 2004), the DJ-1 gene (PARK7) (Bonifati, Rizzu et al. 2003), the LRRK2 gene (PARK8) (Mata, Wedemeyer et al. 2006) and more recently the ATP13A2 gene (PARK9) (Ramirez, Heimbach et al. 2006).

1.3 Parkinson's Disease Pathology and Lewy Bodies

Neurodegeneration in PD is characterized by neuronal loss mainly in the dopaminergic neurons in the Substantia nigra pars compacta (SNpc) in the

central nervous system (CNS). The neuronal loss is accompanied by the presence of cytoplasmic intraneuronal protein aggregates inclusions known as LBs and LNs. LBs and LNs are considered to be the pathological hallmarks of PD, where α -Syn constitutes the major component of these neuronal inclusions in both familial and sporadic PD (Spillantini, Crowther et al. 1998b). Other neurodegenerative diseases have also been found to have the characteristic α -Syn positive inclusions in neuronal or glial cells, and therefore collectively referred to as synucleinopathies. This group of diseases includes Multiple System Atrophy (MSA) (Spillantini, Crowther et al. 1998a; Duda, Giasson et al. 2000), dementias with Lewy bodies (DLB) (Spillantini, Schmidt et al. 1997) and LB variant of AD (Goedert 2001). In addition, some non-neurodegenerative diseases have been reported to contain a considerable percentage of α -Syn positive neuronal inclusions, such as brain iron accumulation type-1 (Arawaka, Saito et al. 1998) and Down's syndrome (Lippa, Schmidt et al. 1999).

The process of neurodegeneration in PD patients occurs initially in other regions of the brainstem, reaching dopaminergic neurons in the substantia nigra at later stages. It starts in the olfactory bulb, dorsal motor vagal nucleus and the locus coeruleus (Braak, Del Tredici et al. 2003). Neurodegeneration may also occur in nerve cells in the peripheral autonomic ganglia (Wakabayashi, Matsumoto et al. 1997). LBs were initially described in 1912 by Frederich Lewy as rounded cytoplasmic inclusions consisting of a translucent glassy core that stains brightly for eosin, surrounded by a pale staining peripheral halo of radiating fibrils of α -Syn and other proteins (Bennett 2005), such as chymotrypsin A, elements of proteasome (Li, Ito et al. 1997), ubiquitin ligase (E3) parkin (Choi, Golts et al. 2001) and some cytoskeletal protein such as tubulin (Galloway, Grundke-Iqbal et al. 1988). Prior to the discovery that α -Syn is the major component of LBs, these proteinaceous inclusions have been marked using ubiquitin antigenicity (Lennox, Lowe et al. 1989).

1.4 Parkinson's Disease Symptoms

PD is a neurological disorder characterized by a large number of motor and non-motor features that have impact on individual's everyday function to varying degree. Resting tremor, bradykinesia, rigidity and loss of postural reflexes are generally considered the cardinal signs of the disease. Other clinical features include secondary motor symptoms such as hypomimia, dysarthria, dysphagia, sialorrhoea, micrographia, shuffling gait, festination, freezing, dystonia and glabellar reflexes. In addition, some non-motor symptoms have been described such as autonomic dysfunction, neurobehavioral abnormalities, sleep disorders and sensory abnormalities like anosmia, paresthesias and pain. The absence of resting tremor along with early occurrence of gait difficulty, postural instability, dementia, hallucinations and the presence of dysautonomia, ophthalmoparesis, ataxia and other atypical features, coupled with poor or no response to levodopa, suggest diagnosis other than PD (Jankovic 2008).

1.5 Alpha Synuclein and Parkinson's Disease

1.5.1 Alpha Synuclein Family and Disease Mutants

1.5.1.1 The Synuclein family

Alpha-synuclein (α -Syn) belongs to a protein family known as the Synuclein family, which includes two other proteins named beta-synuclein (β -Syn) and gamma-synuclein (γ -Syn). α -Syn was first discovered when a protein named synuclein was isolated from the electric organ of *Torpedo californica* using an antiserum against cholinergic vesicles. It was given the name synuclein because of its localization in the presynaptic nerve terminals and also close to the nuclear

envelope of neurons (Maroteaux, Campanelli et al. 1988). A highly homologous protein was later found in rat brains (Maroteaux and Scheller 1991). Subsequent research of AD led to the identification of a peptide called NAC (non-A beta component) in the amyloid plaques of brains from AD patients. The precursor protein of that peptide (NACP) has been shown to be homologous to the rat synuclein protein (Ueda, Fukushima et al. 1993). Furthermore, Jakes and co-workers (Jakes, Spillantini et al. 1994) identified two synuclein-related proteins of 140 and 134 amino acids in length from a cytosolic extract of human brain. The human 140 amino acid protein was found to be identical to the previously reported precursor protein of NAC peptide (NACP) in AD amyloid plaques, and shares high similarity with synuclein from Torpedo and rat brains. This was named α -Syn. Two other isoforms of α -Syn have subsequently been discovered. The 112 and 126 amino acid isoforms are produced by alternative splicing of the gene encoding α -Syn leading to in-frame deletions of exon 5 and exon 3 respectively (Xia, Saitoh et al. 2001; Uversky 2007). The 134 amino acid protein was found to be a homologue of the bovine phosphoneuroprotein 14 (Nakajo, Tsukada et al. 1993) and was named β -Syn (Jakes, Spillantini et al. 1994). The most recently discovered member of the synuclein family, γ -Syn, was isolated and characterized in 1997 (Ji, Liu et al. 1997; Lavedan, Leroy et al. 1998). γ -Syn is a 127 amino acid protein and was found 75.3% similar to the previously described Torpedo protein mainly at the amino-terminal part (Lavedan 1998).

1.5.1.2 Localization and expression of synuclein proteins

The synuclein family members, α -Syn, β -Syn and γ -Syn are encoded by three different genes, SNCA, SNCB and SNCG, which map to chromosomes 4q21.3-q22, 5q35 and 10q23, respectively. All three synucleins are predominantly expressed in the brain. α -Syn and β -Syn expression has been detected primarily in the CNS, particularly in presynaptic nerve terminals in the neocortex, hippocampus, striatum, thalamus, cerebellum, cerebellar cortex, substantia nigra

and brain stem (Jakes, Spillantini et al. 1994; Iwai, Masliah et al. 1995; Irizarry, Kim et al. 1996). Both α -Syn and β -Syn have been shown to be found in the cytosol. γ -Syn is primarily expressed in cell bodies and axons of primary sensory neurons, sympathetic neurons and motor sensory neurons of the peripheral nervous system (Buchman, Hunter et al. 1998). γ -Syn expression has also been reported at moderate levels in the heart, skeletal muscles, and much lower levels in the kidney, liver and pancreas with no expression observed in the lung and placenta (Lavedan, Leroy et al. 1998). It is important to mention that abnormally high expression of γ -Syn has been associated with many types of cancers, such as breast tumours (Jia, Liu et al. 1999), where it has been considered as a marker of tumour progression (Lavedan 1998; Jia, Liu et al. 1999), as well as ovarian tumours (Bruening, Giasson et al. 2000). However, it is not known what role γ -Syn can have in normal breast or ovary tissues, as levels of γ -Syn are almost undetectable at the surface epithelial cells of a normal ovary.

1.5.2 Alpha synuclein structure

Generally speaking, all synucleins are small proteins with calculated molecular mass close to 14kDa, however when run on SDS-PAGE gels synucleins have displayed an apparent molecular mass of 19-20kDa, with α -Syn at 16-17kDa running slightly faster than the others (Jakes, Spillantini et al. 1994).

Several spectroscopic and structural studies, such as NMR, have shown that α -Syn is a disordered protein that belongs to the rapidly growing family of natively unfolded proteins that have little or no ordered structure under physiological conditions (Weinreb, Zhen et al. 1996; Uversky, Li et al. 2001b). The primary sequence of human α -Syn can be divided to three distinct regions: 1) an amino terminal region (residues 1-60) containing four 11-amino acid imperfect repeats with a highly conserved motif KTKEGV, which is responsible for lipid binding

(Perrin, Woods et al. 2000); 2) a hydrophobic centre domain (residues 61-95), also referred to as the non-amyloid component NAC, which contains two additional repeats and is believed to be responsible for α -Syn's ability to form beta-sheet rich fibrils and 3) a highly negatively charged carboxy-terminal region (residues 96-140) (Figure 1.2).

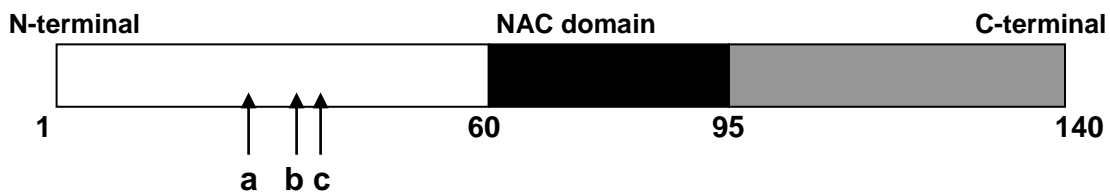


Fig. 1.2 Alpha synuclein schematic representation

α -Syn contains three main domains namely, the amphipathic N-terminal domain, the hydrophobic NAC central domain and the acidic C-terminal domain. Arrows a, b and c represent the three disease related mutations in α -Syn, Ala30Pro, Glu46Lys and Ala53Thr, respectively.

The highly conserved N-terminal domain is a common feature to all synucleins. For α -Syn, this region has been predicted to form amphipathic α -helices structure when associated with lipid membranes (Davidson, Jonas et al. 1998; Perrin, Woods et al. 2000; Eliezer, Kutluay et al. 2001). This conformation is characterized by two helical domains interrupted by a short non helical turn (Davidson, Jonas et al. 1998). This is due to the presence of the consensus imperfect repeats (KTKEGV) in α -Syn that resembles an 11-residue repeating motif found in lipid binding domain of apolipoproteins (George, Jin et al. 1995). It has been suggested that α -Syn adopts α -helical rich structure *in vivo* as well, particularly the membrane- and synaptic vesicles-associated states (Sung and Eliezer 2006). Furthermore, the presence of these repeats in α -Syn, which have unknown function, has also been proposed to play a role in protein-protein interactions (Jakes, Spillantini et al. 1994). These repeats contain clusters of

basic and acidic amino acids and such clusters have been proposed to mediate binding between structural proteins (Weinreb, Zhen et al. 1996; Davidson, Jonas et al. 1998) and dimerization (Jensen, Hojrup et al. 1997).

The central hydrophobic region (NAC) of α -Syn has been implicated in α -Syn aggregation and fibrillation (Giasson, Murray et al. 2001; Uversky, Li et al. 2002b). NAC is one of the main components of amyloid plaques in Alzheimer's brains (Ueda, Fukushima et al. 1993) and can form ordered fibrils *in vitro* (Iwai, Masliah et al. 1995). Moreover, NAC has been shown to seed A β (1-40) amyloidogenesis (Han, Weinreb et al. 1995). In addition, the substitution of amino acid residue at position 76 (Ala) with a charged amino acid (Glu or Arg) results in a decrease in α -Syn association, whereas deletion of amino acid residues 71-82 in human α -Syn prevents protein aggregation (Giasson, Murray et al. 2001). Finally, the N-terminal region of the NAC sequence has been shown to exhibit similarity to the amino acid sequence crucial for aggregation of other amyloidogenic polypeptides such as A β , and prion protein (Gly-Ala-X-X), where X is an amino acid with aliphatic side chain (El-Agnaf, Bodles et al. 1998). These observations together suggest the possible direct involvement of the NAC domain in the pathologic aggregation of α -Syn in neurodegenerative diseases. The C-terminal region of α -Syn has been characterized by its enrichment in acidic amino acid residues (Asp and Glu) and prolines. It also contains three out of four tyrosine residues (positions 125, 133 and 136). The fourth tyrosine is found at position 39. There are no tryptophan residues in α -Syn. The highly acidic C-terminal region has been suggested to possess chaperone like activity (Kim, Paik et al. 2002), and also to regulate α -Syn aggregation (Murray, Giasson et al. 2003). In fact, it has been shown that C-terminally truncated α -Syn assembled into fibrils more rapidly than the full length protein (Crowther, Jakes et al. 1998).

Both β -Syn and γ -Syn share high homology with α -Syn at the amino acid sequence level. Spectroscopic studies have shown that both β -Syn and γ -Syn, in their free state, behave similarly to α -Syn and both are unfolded proteins (Uversky, Li et al. 2002b). Using amino acid sequence alignment, β -Syn has been found to be 61% identical to α -Syn. Most of this homology is observed in the amino terminal half, suggesting that this may serve a function shared between α -Syn and β -Syn (Jakes, Spillantini et al. 1994). γ -Syn has been found to be 50% identical to α -Syn (Figure 1.3).

	1	10	20	30	40
(α -Syn)	MDVEMKGL	<u>SKAKEGVVAAA</u>	<u>EKTKQGVAAEA</u>	<u>GKTKEGVLYVG</u>	
(β -Syn)	MDVEMKGL	<u>SMAKEGVVAAA</u>	<u>EKTKQGVTEAA</u>	<u>EKTKEGVLYVG</u>	
(γ -Syn)	MDVE KKGF	<u>SIAKEGVVGAV</u>	<u>EKTKQGVTEAA</u>	<u>EKTKEGVMYVG</u>	
	50	60	70	80	
	<u>SKTKEGVVHGV</u>	ATVA <u>EKTKEQVTNVG</u>	GAVVTGVTAVA	<u>QKTVEGAGSIA</u>	
	<u>SKTREGVVQGV</u>	ASVA <u>EKTKEQASHLG</u>	GAVFSG-----	--AGNIA	
	<u>AKTKENVVQSV</u>	TSVA <u>EKTKEQANAVS</u>	EAVVSSVNTVA	<u>TKTVEEAENIA</u>	
90	100	110	120	130	140
AATGFVKKDQLGKN-----EEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA					
AATGLVK REEFPTDLKPEEVAQEAAEPLIEPLMEPEGESYEDPPQEEYQEEYEPEA					
VTSGVVRKEDLRPSAPQQEGVASKEKEEVAEEAQSGGD					

Fig. 1.3 Sequence alignment of human α -Syn, β -Syn and γ -Syn.

Underlined is the consensus imperfect hexameric repeat (KTKEGV) presence in the N-terminal region of the synucleins. Spaces (-) represents the 11 amino acids absence from β -Syn sequence in the NAC domain. Bold characters indicate a differences from the α -Syn sequence.

Compared to α -Syn and γ -Syn, β -Syn lacks 11 amino acid residues in the central highly hydrophobic region, and has an increased helical propensity which might stabilize its disordered native state and explain why β -Syn has been found to have reduced propensity to self-aggregate and form oligomers and fibrils (Hashimoto, Rockenstein et al. 2001; Uversky, Li et al. 2002b; Bertoncini, Rasia et al. 2007). However, at its C-terminal region, β -Syn has three tyrosine residues located identically to α -Syn (Uversky, Li et al. 2002b).

In this regard, it is important to mention that two of the disease-related mutants of α -Syn (Ala30Pro and Ala53Thr) were found to have no effect on the conformational and structural properties of the monomeric protein (Conway, Harper et al. 1998; Li, Uversky et al. 2001). Moreover, it has been shown that the propensity of α -Syn to form α -helical structures was reduced by both mutants (Ala30Pro and Ala53Thr), whereas the beta-sheet content was predicted to be slightly enhanced (Li, Uversky et al. 2001).

1.5.3 Alpha synuclein function

α -Syn has been estimated to account for as much as 1% of total protein in soluble cytosolic brain fractions (Iwai, Masliah et al. 1995). It is surprising that the function of such a highly abundant protein is poorly understood. It has been shown that in presynaptic terminals, monomeric α -Syn exists in equilibrium between two forms, free and plasma membrane- or vesicle-bound (McLean, Ribich et al. 2000). Approximately 15% of α -Syn has been revealed to be membrane-bound (Lee, Choi et al. 2002). Based on that association with vesicular structures, several studies suggested a role of α -Syn in membrane-associated processes at the presynaptic terminals, such as regulation of synaptic neurotransmission and dopamine (DA) release (Abeliovich, Schmitz et al. 2000). In addition, α -Syn may regulate the size of synaptic vesicle pool in mature

neurons, as deletion of α -Syn from cultured hippocampal neurons resulted in decreased synaptic vesicle pool as demonstrated by electron microscopy (Murphy, Rueter et al. 2000). α -Syn also interacts with synaptic phospholipid vesicles that contain acidic phospholipids (Davidson, Jonas et al. 1998), as well as various phospholipid membranes (Jo, McLaurin et al. 2000) and biological and general cellular membranes (Jensen, Nielsen et al. 1998; McLean, Kawamata et al. 2000). Moreover, α -Syn has been suggested to modulate the organization of membrane lipid components by affecting the integrity of the membranes' bilayers and causing the formation of non-bilayer or small vesicular structures (Madine, Doig et al. 2006). α -Syn has been shown to inhibit lipid oxidation when bound to lipid membranes, which has been attributed to its easy oxidation via the formation of methionine sulfoxide (Zhu, Qin et al. 2006).

One of the first physiological functions anticipated for α -Syn is to regulate the activity of several enzymes. For example, α -Syn has been shown to act as selective inhibitor of phospholipase D2 (PLD2), which is an active isoform of the phospholipase D enzymes, and is localized primarily along plasma membrane and hydrolyses phosphatidylcholine to phosphatidic acid (Jenco, Rawlingson et al. 1998). In addition, α -Syn has also been shown to inhibit tyrosine hydroxylase (TH), which is the rate limiting enzyme in dopamine synthesis, thus supporting the hypothesis that α -Syn is involved in the regulation of synaptic neurotransmission (Perez, Waymire et al. 2002). However, the precise function of α -Syn is not yet determined and research on α -Syn continues to propose possible functions of the protein in biological processes. Indeed, α -Syn has been suggested to interact with many proteins and ligands, and to act as a molecular chaperone assisting in the process of folding and refolding of synaptic proteins (Chandra, Gallardo et al. 2005). This proposed function was further emphasized when α -Syn was shown to bind to the cytoplasmic chaperone 14-3-3, which was reported to bind and activate TH (Ostrerova, Petrucelli et al. 1999; Perez, Waymire et al. 2002). In addition, α -Syn has been suggested to play a role in the

protection of the nerve terminals via its cooperation with CSP α , an abundant synaptic vesicle protein, which possesses a co-chaperone function and is essential for neuronal survival (Chandra, Gallardo et al. 2005). The accomplishment of this cooperation was suggested to require phospholipid binding by α -Syn (Chandra, Gallardo et al. 2005). Finally, a role of α -Syn in inhibiting the endoplasmic reticulum to Golgi vesicular trafficking has been shown, with an impairment of endoplasmic reticulum-associated degradation (Cooper, Gitler et al. 2006).

1.5.4 Metal binding

PD is thought to be of multi-factorial aetiology, suggesting both genetic and environmental factors. Taking into account the potential role of α -Syn in the disease pathology, many studies have addressed the possible involvement of α -Syn in the environmental causation theory. A range of environmental factors and neurotoxins have been associated with PD, which directly interact with α -Syn, including metal ions, pesticides, herbicides and organic solvents. Interaction of α -Syn with these neurotoxins provoked the aggregation of α -Syn *in vitro* (Uversky, Li et al. 2001c; Uversky, Cooper et al. 2002) and was associated with increased α -Syn positive inclusions in animal models (Betarbet, Sherer et al. 2000; Sherer, Kim et al. 2003). In this respect, metals have been recognized as risk factors in several neurodegenerative diseases, such as AD (Huang, Atwood et al. 2004) and Prion disease (Brown, Guantieri et al. 2004). Less information is known about the relationship between metals and PD (Zayed, Ducic et al. 1990; Gorell, Johnson et al. 1997). Most recent studies propose a correlation between the exposure to heavy metals and PD development (Gorell et al. 1997; Gorell et al. 1999; Weisskopf et al. 2010; Bonilla-Ramirez et al. 2011).

Metals serve vital roles in many biological systems. They do not only represent factors that can stabilize and modulate protein structures, but are also essential survival elements as they participate in various biologically important reactions, such as DNA and RNA synthesis, redox reactions and act as co-factors to many enzymes (Regan 1995; Sayre, Moreira et al. 2005). The latter property is exhibited by both redox active (copper and iron) and redox inactive (zinc) metal ions. Therefore, disruption of metal ion homeostasis can be harmful as their deficiency can lead to disturbances in central nervous system and other organs' function, while their accumulation can be toxic (Sayre, Moreira et al. 2005). Due to the general acceptance that oxidative stress might be involved in the pathogenesis of neurodegenerative diseases such as AD and PD (Paik, Shin et al. 2000), redox active metal ions such as copper and iron are of interest. These metals are implicated in the disease pathology affecting areas of the brain in both PD and AD. The brains of patients suffering with these diseases have been shown to contain abnormally high levels of such metals, particularly iron (Sayre, Moreira et al. 2005). Post-mortem analysis of PD brains provided information about the involvement of metals in PD, where increased levels of copper have been reported in cerebrospinal fluid of PD patients (Pall, Williams et al. 1987). High levels of zinc were found in the substantia nigra of PD brains (Dexter, Wells et al. 1989). Moreover, iron deposits have also been identified as components of Lewy bodies (LBs), in addition to elevated levels of aluminium in the Parkinsonian substantia nigra (Hirsch, Brandel et al. 1991). An understanding of the outcome of increased levels of metals in the brain come from animal experiments, for example an injection of FeCl_3 into the substantia nigra of adult rats caused a selective decrease in the level of dopamine, and resulted in impaired dopamine-related behavioural responses (Youdim, Ben-Shachar et al. 1991).

In vitro experiments have shown that a number of mono-, di- and trivalent metal ions can facilitate α -Syn oligomerization and aggregation, particularly copper

(Cu²⁺), as assessed by laddering on SDS-PAGE gels (Paik, Shin et al. 1999) and using the Thioflavin-T based fluorometric assay (Uversky, Li et al. 2001b). For example, aluminium has been shown to cause alterations in α -Syn structure, such as transition into a secondary structure with 33% more α -helical content (Paik, Lee et al. 1997) and induced fibril formation (Uversky, Li et al. 2001b). Given the effect of metals on accelerating α -Syn aggregation and fibril formation, much research has been conducted to assess the ability of the protein to bind metals. Although it remained controversial for a long time, it is becoming clear how and where metals (especially copper and iron) can bind to α -Syn. Early studies have shown that Cu²⁺ binds to the C-terminal domain of α -Syn and induces its oligomerization (Paik, Shin et al. 1999). A single molecule of α -Syn can bind 10 molecules of copper with a dissociation constant of 59 μ M. However, subsequent studies have revealed that the primary Cu²⁺ binding site is located at the N-terminal domain involving residues 1-9 and residues 48-52, with the Histidine residue at position 50 being an anchoring residue (Rasia, Bertoncini et al. 2005; Binolfi, Rasia et al. 2006). Rasia et al. 2005 have shown that α -Syn strongly binds two Cu²⁺ ions per monomer at its N-terminal copper-binding interface with dissociation constant of 0.1-50 μ M and that this binding is independent of the presence of the C-terminal interface. They also suggested that the C-terminal interface involving residues 119-123 is able to coordinate more Cu²⁺ but with significantly lower affinity. A similar result has also been reported showing a high affinity binding site (0.1 μ M) at the N-terminal of α -Syn, and a low affinity metal binding site at the C-terminal (Binolfi, Rasia et al. 2006). Finally, in a more recent study convincing evidence suggests that there are two independent, non-interacting copper-binding sites with significantly different binding affinities at the N-terminal region of α -Syn (Binolfi, Lamberto et al. 2008). They demonstrated the involvement of the Methionine residue at position 1 as the primary anchoring residue for Cu²⁺ binding. While a second Cu²⁺ binding site with lower affinity is centred at the Histidine residue at position 50. The presence of two copper binding sites in α -Syn has also been reported by a different group (Bharathi and Rao 2007), where they also showed that α -Syn has a single Fe³⁺

binding site. The specificity of iron binding to α -Syn is still elusive. However, the C-terminal domain of α -Syn, specifically residues 110-140 centring on Asp121, has been proposed to be able to bind Fe^{2+} , in addition to other divalent metal ions such as Mn^{2+} , Co^{2+} and Ni^{2+} (Binolfi, Rasia et al. 2006).

The mechanisms by which metals interact with the proposed metal-binding sites are still not clear. Nevertheless, the preferential interaction with the acidic C-terminus is suggested to be due to the negatively charged surface (Paik, Shin et al. 1999). It has also been hypothesized that copper binding to the N-terminus of α -Syn would perturb the long range interactions critical for stabilizing a soluble auto-inhibited conformation of α -Syn (Rasia, Bertoncini et al. 2005).

1.5.5 Alpha synuclein aggregation

Since fibrillar α -Syn is the hallmark of PD and other synucleinopathies, it is likely that fibrillation plays a critical role in the disease aetiology and progress. Identifying conditions that lead to α -Syn fibrillation are fundamentally important for the design of novel therapies. It has been established that recombinant α -Syn and its disease mutant forms are able to assemble into filaments with morphologies and staining properties similar to those extracted from disease affected brains (El-Agnaf, Jakes et al. 1998b; Uversky, Li et al. 2001b; Fredenburg, Rospigliosi et al. 2007). This is important as it verifies the use of recombinant protein as a model system for analysing α -Syn aggregation *in vitro*. Different final products with distinct morphologies may occur from α -Syn aggregation depending on the experimental conditions, and this includes fibrils, soluble oligomers and insoluble amorphous aggregates (Fink 2006). Research has aimed to identify conditions that would induce the formation of the pathologic insoluble aggregates of α -Syn. It has been shown that α -Syn has a strong tendency to aggregate and it does so spontaneously (Hashimoto, Hsu et al.

1998). Monomeric α -Syn, when incubated at 37°C and neutral pH, has been shown to form fibrils in a condition-dependent manner with agitation greatly enhancing the process (Fink 2006). On the other hand, incubating α -Syn at acidic pH leads to a shift in the resultant aggregate morphology towards the formation of amorphous aggregates rather than fibrils (Hoyer, Antony et al. 2002).

α -Syn aggregation kinetics *in vitro* show sigmoidal curves defined by an initial lag phase, followed by exponential phase (fibril growth phase) and final plateau after fibrils are completely formed. α -Syn aggregation has been proposed to display a nucleation dependent mechanism where a partially folded intermediate is thought to be critical for α -Syn oligomerization and fibrillation (Uversky, Li et al. 2001a; Rasia, Bertocini et al. 2005). However, it remains a fundamental question as to what forces and factors causing α -Syn transformation from random coiled to a structured form with predominantly β -pleated sheet structure (Uversky, Li et al. 2001a). A model has been proposed for α -Syn fibrillation pathway. The monomeric unfolded protein undergoes a conformational change into an aggregation-prone intermediate, which can then lead to the formation of one of the final end products mentioned earlier (fibrils, amorphous aggregates and soluble oligomers) (Uversky, Li et al. 2001a; Fink 2006). This model is further supported by research which showed a correlation between partial folding and fibrillation (Uversky, Li et al. 2001a). Consequently, factors that favour the formation of the partially folded intermediate will facilitate fibril formation.

It has been well demonstrated that a variety of different endogenous and exogenous factors and ligands, as well as certain *in vitro* conditions, stimulate a conformational change that results in modulation of α -Syn aggregates morphology and kinetics. Partial folding has been considered and shown to represent the main mechanism by which α -Syn aggregation is induced. In this respect, several commonly used pesticides (rotenone, paraquat) and some di-

and trivalent metal ions (Cu^{2+} , Fe^{3+} and Al^{3+}) have been shown to cause significant alterations in the rate of α -Syn fibril formation (Paik, Shin et al. 1999; Uversky, Li et al. 2002a). Their effectiveness has been shown to be correlated with their ability to induce and stabilize a fibrillation-prone partially folded intermediate, and with increased ion charge density in the case of metals (Uversky, Li et al. 2001b; Uversky, Li et al. 2001c; Uversky, Li et al. 2002a). However, the mechanism by which metals act has been further proposed to involve metal binding to the negatively charged carboxylates, thus masking the electrostatic repulsion and facilitating collapse to partially folded conformation. The identification of specific metal binding sites for some metals supports this mechanism of action (Uversky, Li et al. 2001b; Uversky, Li et al. 2002a). Protein concentration and molecular crowding also enhance α -Syn fibril formation via the partial folding mechanism (Uversky, Cooper et al. 2002). α -Syn has been proposed to be present in an equilibrium between its unfolded and partially folded states (Uversky 2007), and *in vitro* experiments have shown that high concentrations of α -Syn result in increased fibrillation rate which is likely to be due to elevated intermediate concentration (Uversky, Li et al. 2001b). This is important in the understanding of the pathogenesis of Parkinson's disease, particularly in the case of patients with the triplication of the α -Syn locus, which leads potentially to an elevated level of α -Syn formation (Singleton, Farrer et al. 2003; Pals, Lincoln et al. 2004) and therefore increasing protein concentration and partial folding. In addition, the presence of high concentration of macromolecules is a common property of cells where 25% of the volume of a standard cell consists of macromolecules such as inert proteins, nucleic acids and carbohydrates (Minton 2001; Fink 2006). This can have major consequences on the properties of macromolecules, such as protein-protein interactions. In relation to α -Syn aggregation, this was investigated *in vitro* with high concentration of various types of biopolymers causing acceleration of α -Syn fibrillation in a concentration dependant manner (Shtilerman, Ding et al. 2002; Uversky, Cooper et al. 2002). Finally, C-terminally truncated α -Syn was found in Lewy bodies (LBs) along with full length protein and was found to be more prone

to fibrillate (Murray, Giasson et al. 2003). The effect of post-translational modification on α -Syn fibrillation has also been investigated. Pathogenic inclusions have also been shown to have extensively phosphorylated α -Syn at Ser129 (Fujiwara, Hasegawa et al. 2002; Nishie, Mori et al. 2004). Although α -Syn under normal conditions has been reported to be non-phosphorylated, specific phosphorylation of α -Syn at residue Serine at position 129 resulted in accelerated oligomerization and fibrillation (Fujiwara, Hasegawa et al. 2002).

Besides the continuous interest in identifying factors contributing to increased α -Syn aggregation, extensive research has been also directed towards specifying factors that can inhibit α -Syn aggregation. Modifying α -Syn by methionine oxidation or tyrosine nitration has been shown to completely inhibit α -Syn fibril formation due to the formation of stable oligomers (Uversky, Yamin et al. 2002; Yamin, Uversky et al. 2003; Uversky, Yamin et al. 2005). This is crucial as the existence of nitrated α -Syn in Lewy bodies (LBs) has been demonstrated (Duda, Giasson et al. 2000; Giasson, Duda et al. 2000). Oxidative stress has also been implicated in Parkinson's disease, particularly taking into consideration alteration in iron homeostasis. Increased iron levels, which is a redox active metal, can cause increased generation of free radicals via Fenton reaction which in turn can oxidatively damage proteins including α -Syn (Hashimoto, Hsu et al. 1999).

In contrast to α -Syn, β -Syn does not fibrillate when incubated under the same conditions as α -Syn (Uversky, Li et al. 2002b). However, it has been shown that β -Syn can be forced to fibrillate under specific conditions, such as presence of metals, pesticides, molecular crowding (Yamin, Munishkina et al. 2005). Interestingly, fibrillation of α -Syn was completely inhibited at 4:1 molar excess of β -Syn or γ -Syn over α -Syn (Uversky, Li et al. 2002b). This suggests that both β -Syn and γ -Syn may behave as negative regulators of α -Syn fibrillation (Uversky, Li et al. 2002b). β -Syn was also shown to inhibit α -Syn fibrillation in animal

models (Hashimoto, Rockenstein et al. 2001). The mechanism is not clear yet, but it was suggested to be mediated by stabilizing α -Syn aggregates via direct interaction (Uversky, Li et al. 2002b) or even via affecting and modulating α -Syn expression level (Fan, Limprasert et al. 2006). Importantly, these observations suggest that the balance in the levels of the synuclein family members may be essential for disease prevention. Finally, since dopaminergic neurons in the substantia nigra are the most susceptible to neurodegeneration in PD, and dopamine is being sensitive to oxidation, this has led to the suggestion that dopamine is linked in a way to the disease pathology (Uversky 2007). It has been shown that dopamine can inhibit α -Syn fibril formation *in vitro* (Conway, Rochet et al. 2001; Li, Zhu et al. 2004). The mechanism is not well known, but it was attributed to the covalent modification of α -Syn by o-quinones derived from dopamine leading to the production of modified proto-fibrils that are unable to fibrillate (Conway, Rochet et al. 2001).

1.5.6 Toxicity of Alpha Synuclein

The presence of α -Syn aggregates in LBs is a common feature of many neurodegenerative diseases, including PD. It is still not clear whether cell death is the result of α -Syn aggregation. This prompted investigations towards the assessment of α -Syn toxicity both *in vitro* cell-culture and animal models, and it was shown to be toxic in many studies (El-Agnaf, Jakes et al. 1998a; Sung, Kim et al. 2001). The mechanism underlying α -Syn toxicity and which species are responsible for the neuronal death are still not clear. As surviving neurons in the brain contain aggregates (fibrils), then this could be an indication that other aggregate species might be involved. Therefore, specific populations of α -Syn may be responsible for the toxicity, and a growing body of evidence suggest that prefibrillar oligomers and proto-fibrils rather than mature fibrils are the toxic species (Lashuel, Petre et al. 2002; Danzer, Schnack et al. 2007; Wright, Wang et al. 2009). Evidence for this hypothesis has been confirmed using an *in vitro*

cell model of human dopaminergic neurons, wherein toxicity was observed with non-fibrillar, small α -Syn aggregates, suggesting that soluble species mediate toxicity (Xu, Kao et al. 2002). This has also been proposed to be the case with intermediates formed during amyloid assembly of A β -peptide in Alzheimer's disease (Lashuel, Hartley et al. 2002).

The mechanism of oligomer toxicity is not clear, however, there are many hypotheses. The formation of oligomers has been shown to increase leakiness of synthetic lipid vesicles (Volles, Lee et al. 2001) and therefore might cause damage to cellular membranes *in vivo*. The mechanism by which α -Syn oligomers damage cellular membranes is not characterized, but it could be mediated by integrating to the membrane and forming pore-like structures which could cause membrane permeability (Volles and Lansbury 2002). This might lead to altered ion balance such as elevation of intracellular calcium levels (Danzer, Haasen et al. 2007). Calcium disruption induced-toxicity was a common proposed mechanism caused by other amyloidogenic peptides (amyloid β peptide, prion) (Demuro, Mina et al. 2005). However, cell culture studies of α -Syn toxicity are still controversial. Some studies have shown that expression of α -Syn or its disease mutants is associated with aggregate formation (Ostrerova-Golts, Petrucelli et al. 2000; Lee, Hyun et al. 2001) and inducing the cells to be more vulnerable to cellular changes (Zhou, Yap et al. 2006). On the other hand, other studies have shown that α -Syn expression can be protective against cellular stresses (Hashimoto, Hsu et al. 2002). Also, α -Syn oligomers generated *in vitro* and added extra-cellularly to cultured cells have been shown to cause toxicity (Liu, Giasson et al. 2005; Wright, Wang et al. 2009). Finally, expression of α -Syn in yeast has also been reported to form cytoplasmic inclusions that are associated with toxicity (Soper, Roy et al. 2008).

1.6 Regulation of Alpha Synuclein Expression

Several lines of evidence suggest the involvement of α -Syn gene SNCA in Parkinson's disease. Multiple point mutations (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998) as well as gene multiplications in the α -Syn gene SNCA (Singleton, Farrer et al. 2003; Pals, Lincoln et al. 2004), have been associated with cases of familial and sporadic PD. However, the majority of PD patients do not have gene mutations or multiplications, yet they show α -Syn deposits in Lewy bodies (LBs) (Mezey, Dehejia et al. 1998). Therefore, it can be assumed that there are other mechanisms contributing to α -Syn role in PD pathogenesis. Studies have demonstrated that over-expression of WT α -Syn, in cases of SNCA multiplications, is sufficient to cause Parkinsonism in a dose dependent manner (Singleton, Farrer et al. 2003; Pals, Lincoln et al. 2004). For example, carriers of the three SNCA copies (patient with gene duplication) were shown to have 1.5-fold increase in α -Syn mRNA and protein levels, whereas carriers of the four SNCA copies (patients with gene triplication) were shown to have 2-fold increase (Pals, Lincoln et al. 2004). To some degree, this is consistent with the observation that molecular crowding leads to dramatic acceleration of α -Syn aggregation and fibrillation *in vitro* (Shtilerman, Ding et al. 2002; Uversky, Cooper et al. 2002; Fink 2006). Also, several model systems have been studied in order to better illustrate the role of α -Syn in PD progression. For instance, transgenic mice over-expressing α -Syn were shown to display neuronal inclusions that are similar to Lewy bodies LBs, increased degradation of dopaminergic neurons and motor deficits (Masliah, Rockenstein et al. 2000). In addition, the expression of WT, A53T and A30P α -Syn in transgenic flies resulted in similar effects such as neuronal inclusions and motor deficits (Feany and Bender 2000). Hence, over-expression of α -Syn could play a similar role in disease aetiology and it is important to understand the regulation of α -Syn expression and factors that can affect it.

Differences in the expression levels of α -Syn might not only be dependent on disease status but also might be modified by genetic variability (Fuchs, Tichopad et al. 2008). Several studies have shown that genetic variability in α -Syn gene (promoter and 3' region) can modulate the risk to develop sporadic PD by affecting α -Syn expression level (Pals, Lincoln et al. 2004; Fuchs, Tichopad et al. 2008). The best characterised genetic variation associated with PD is the NACP-Rep1 polymorphic alleles (Kruger, Vieira-Saecker et al. 1999; Maraganore, de Andrade et al. 2006). NACP-Rep1 is a polymorphic nucleotide complex repeat site located ~10kb upstream of the transcription start site of α -Syn gene SNCA (Xia, Rohan de Silva et al. 1996; Touchman, Dehejia et al. 2001). It has been demonstrated that NACP-Rep1 has a reproducible effect on regulating transcriptional activity, where a fragment of SNCA promoter including different alleles of NACP-Rep1 repeat, led to differences in expression levels (Chiba-Falek and Nussbaum 2001; Chiba-Falek, Touchman et al. 2003). Five alleles of NACP-Rep1 have been identified which differ in size and length by two nucleotides (Xia, Rohan de Silva et al. 1996). Three of those alleles were found to be the most common alleles, (257,259,261bp as measured by length), and allele length variability has been shown to be associated with altered expression of α -Syn gene SNCA (Chiba-Falek and Nussbaum 2001; Chiba-Falek, Touchman et al. 2003). Since the level of α -Syn is directly linked to disease progression, it is important to characterise and identify the α -Syn promoter (enhancer) region.

Importantly, it was reported that levels of α -Syn mRNA increases and β -Syn mRNA decreases in PD brains compared to controls (Rockenstein, Hansen et al. 2001), which is the opposite to normal aging brains (Li, Lesuisse et al. 2004). *In vitro* studies confirming the inhibitory effect of β -Syn over α -Syn, suggest that both α -Syn and β -Syn transcription is regulated to ensure that β -Syn will prevent α -Syn aggregation and disease progression. Therefore, these observations emphasize the importance of a balanced regulation of both α -Syn and β -Syn. Moreover, α -Syn was found to bind metals (Binolfi, Rasia et al. 2006). Metals

such as copper and iron were found to accelerate its aggregation *in vitro* (Uversky, Li et al. 2001b). This is also likely to occur *in vivo*, as metals (zinc, aluminium and copper) were also reported to have elevated levels in brains with normal aging and patients with PD (Pall, Williams et al. 1987; Dexter, Wells et al. 1989; Hirsch, Brandel et al. 1991). Since metals, such as copper, have been found to regulate the expression of other proteins involved in neurodegenerative diseases, such as PrP in Prion disease (Varela-Nallar, Toledo et al. 2006a), metals may have a role in regulating α -Syn expression.

1.7 Aims of project

This project examined the role of alpha synuclein in PD in terms of its relation to metals such as copper and iron. This work aims to:

1. Determine the ferric iron (Fe^{3+}) binding properties to α -Syn including number of binding sites and affinity values.
2. Assess the ability of α -Syn to reduce ferric iron in the presence of copper, based on the protein's binding properties to both metals.
3. Assess the effect of metals on inducing the aggregation of α -Syn and its metal-binding mutants.
4. Explore any effects of metals on regulating α -Syn expression by looking at changes in promoter activity.

Chapter two: Material and Methods

2.1 Materials

All media and reagents were purchased from Sigma-Aldrich (Poole, UK) laboratories unless otherwise stated.

Plasmid Constructs

Plasmid vectors used in recombinant protein production are pET15b and pTrcHis vectors supplied by Novagen and Invitrogen, respectively. The coding sequence of human α -Syn and its mutants were cloned into XhoI /KpnI in pTrcHis vector and into XhoI /BamHI in pET15b vector. pET15b was used for expression of N-terminal (His)₆-tagged proteins in *E.Coli* followed by a thrombin cleavage site. pTrcHis was used for expression of N-terminal (His)₆-tagged proteins in *E.Coli* followed by an enterokinase cleavage site. pET11a and pET3a were used for expression of untagged proteins in *E.Coli*.

pcDNA3.1(-) was obtained from Invitrogen. It is a vector used for transfections to express untagged proteins in mammalian neuroblastoma SHSY-5Y cells. The open reading frame of α -Syn was cloned between KpnI /XhoI sites in the plasmid. Site directed mutagenesis using PCR and paired oligonucleotide primers was used to introduce point mutations into the plasmid.

pGL3-Basic vector was obtained from Promega. It is a Luciferase Reporter Vector that was used for quantitative analysis of factors that regulate mammalian gene expression.

Genomic α -Syn and β -Syn were obtained from cDNA libraries (RPI-27M7 and RZPD 1.0, respectively) (ImaGenes).

Oligonucleotide primers Used

Oligonucleotides used to produce His-tagged and untagged forms of both WT and disease mutant and metal binding mutants of α -Syn, were already available in the laboratory. Oligonucleotides used to introduce mutations in α -Syn and produce proteins used in this project include:

His-tagged Δ 1-9 α -Syn:

F 5' CGC GGC AGC CAT ATG AAG GCC AAG GAG GGA G- 3'
R 5' CTC CCT CCT TGG CCT TCA TAT GGC TGC CGC G- 3'

Ala30Pro α -Syn:

F 5' GGC AGA AGC ACC AGG AAA GAC 3'
R 5' GTC TTT CCT GGT GCT TCT GCC 3'

Ala53Thr α -Syn:

F 5' GTG GTG CAT GGT GTG ACA ACA GTG GCT GAG AAG 3'
R 5' CTT CTC AGC CAC TGT TGT CAC ACC ATG CAC CAC 3'

Glu46Lys α -Syn:

F 5' GCT CCA AAA CCA AGA AGG GAG TGG TGC ATG 3'
R 5' CAT GCA CCA CTC CCT TCT TGG TTT TGG AGC 3'

Δ 2-9 α -Syn:

F 5' GAAGGAGATATACATATGAAGGCCAAGGAGGGAGTTG 3'
R 5' CAACTCCCTCCTTGGCCTTCATATGTATATCTCCTTC 3'

Δ 119-126 α -Syn:

F 5' CTGGAAGATATGCCTGTGATGCCTTCTGAGGAAGGG 3'
R 5' CCCTTCCTCAGAAGGCATCACAGGCATATCTTCCAG 3'

Promoter fragment β -Syn (-3.7/1)

F 5' –ATA TCT CGA GGC ACG CAC CTG TAG TCT C- 3'

R 5' –TAA AGG ATC CGA ACA CGT CCA TCC TGG C- 3'

PCR Mutagenesis

PCR enzymes such as Pwo polymerase (Roche applied science, Burgess Hill, UK) or Prime Star DNA polymerase (TaKaRa), dNTP's and assay buffers were supplied by New England Biolabs Inc (Hertfordshire, UK).

Primers were produced by MWG (Germany).

Transformations

E.Coli XL-2 blue cells were supplied from Stratagene (Oxford, UK). The pET plasmid DNA was used to transform XL-2 blue competent cells, from which permanent bacterial stocks were prepared in 15% sterile glycerol. Protein expression was conducted by DNA transformation into BL21 cells (Promega Ltd, Hampshire, UK).

DNA Purification

DNA purification kits were purchased from QIAGEN and Fermentas Molecular Bio (York, UK).

Agarose Gel Electrophoresis

10X Bluejuice loading dye and 1kb gene ladder were obtained from Invitrogen.

Agarose and ethidium bromide were supplied by Melford Laboratories Ltd (Ipswich).

Protein Expression and Purification

LB broth and Carbenicillin were obtained from Melford Laboratories Ltd (Ipswich).

Isopropyl β -D-1-thiogalactopyranoside (IPTG) was obtained from Apollo Scientific.

Chelating sepharose was purchased from GE healthcare.

Ultra filtration equipment, including filters, was purchased from Millipore.

Dialysis membrane was obtained from Medicell and EDTA from Fisher Scientific (Loughborough).

Tris and Sodium Chloride were obtained from Fisher Scientific.

MOPS was obtained from Sigma-Aldrich.

Phenylmethanesulphonylfluoride (PMSF) was purchased from Melford Laboratories Ltd (Ipswich).

Protein Determination

Biorad reagent was obtained from BioRad Laboratories (Hercules, CA, USA).

Bovine Serum Albumin BSA was obtained from Fisher Scientific.

Protein Purity

SDS gels: 30% acrylamide protogel was purchased from Fisher Scientific.

Ammonium Persulphate (APS) was purchased from Melford Laboratories Ltd (Ipswich).

N,N,N,N-Tetramethylethylene-diamine (TEMED)

Sodium Dodecyl Sulphate (SDS) was obtained from Melford Laboratories Ltd (Ipswich).

Glycine, Coomassie brilliant blue, Methanol and glacial acetic acid were obtained from Fisher Scientific.

Western Blotting

Marvel dry semi-skimmed milk.

PVDF membrane was purchased from Millipore (Bedford, MA, USA).

Blotting paper was purchased from Fisher Scientific.

Tween 20 was purchased from Fisher Scientific.

Antibodies for α -Syn:

Mouse primary antibody directed against the C-terminus of α -Syn (Zymed laboratories).

Sheep primary α -Syn antibody (Abcam) directed against amino acids 11-26 of α -Syn sequence.

Cell Culture

Dulbecco's modified eagles media (DMEM) was obtained from Lonza.

Fetal Bovine Serum (FBS) was obtained from Lonza.

Trypsin-EDTA was obtained from Lonza.

2.2 Methods

2.2.1 Site-directed mutagenesis

Site directed mutagenesis was performed to create mutant form of α -Syn lacking the potential metal binding sites. The mutation was introduced to the plasmid (pET15b) containing α -Syn coding sequence using PCR. The following reactions were set up in 0.5ml thin-wall PCR tubes: 1 μ l template DNA (50-100ng), 1.2 μ l of 25 μ M of each primer, 0.5 μ l of 25mM of dNTPs, 5 μ l 10x buffer (-MgSO₄), 0.5 μ l Pwo Polymerase, 2.5mM MgSO₄ and up to 50 μ l sterilised MilliQ water.

The PCR programme, utilising a Thermo Hybaid PCR Sprint thermal cycler, was as follows: one cycle: 95°C for 30 seconds; 5 cycles: 95°C for 30 seconds, 50°C for 1 minute, 72°C for 7 minutes; followed by 15 cycles: 95°C for 30 seconds, 55°C for 1 minute, 72°C for 7 minutes; finally, one cycle: 72°C for 10 minutes. Temperature was held at 4°C. PCR samples were then Dpn1 treated for one hour at 37°C to remove the remaining parental DNA and transformed afterwards into competent cells (XL-2 blue competent cells). The following are the primer sequences used to create the N-terminal mutation deleting amino acids 1 to 9:

Forward primer: 5' cgcggcagccatatgaaggccaaggaggag 3'

Reverse primer: 5' ctccctccttggccttcatatggctgccgcg 3'

After transformation, single colonies were picked individually and grown in 5ml LB with relevant antibiotic (50µg/ml Carbenicillin). DNA was extracted and 15µl was sent to Geneservice for sequencing.

2.2.2 Preparation of Bacterial Competent Cells

XL-2 Blue Ultra competent cells (Stratagene) and BL21 (DE) competent cells (Promega) were plated on two separate LB agar plates and incubated overnight at 37°C. A single colony from each plate was inoculated into 10ml LB medium and incubated overnight at 37°C. The 10ml overnight culture was then added to 200ml LB medium and incubated at 37°C shaking at 200rpm until it reached an optical density of 0.6 at 600nm. After that, the culture was centrifuged at 4000rpm at 4°C for 10min. Cell pellets were then resuspended in 200ml ice cold 0.1M sterile MgCl₂. The mixture was then centrifuged as mentioned earlier and the cells pellets were resuspended in 100ml ice cold 0.1M CaCl₂. The mixture was incubated on ice for 20min and centrifuged as previous. The cell were resuspended in 10ml ice cold 0.1M MgCl₂ with 15% glycerol and dispensed into 200µl aliquots in 1.5ml tubes sitting on dry ice. The cells were stored at -80°C. All the work was performed next to the flame and all the solutions and tubes were sterilized by autoclaving.

2.2.3 Transformation

XL-2 blue, JM109 and BL21 competent cells were used. Cells were thawed on ice by gently flicking the tube. 3µl of DNA was then added to 50µl of competent cells in pre-chilled tubes and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for one minute and then placed back on ice for another two

minutes. 300µl LB broth was added to the cells/DNA mixture and incubated for 30-60 minutes at 37°C, shaking at 200 rpm. The mixture was centrifuged for 5 minutes at 4000rpm. The cell pellet was then resuspended in 150µl supernatant and plated on LB agar plates supplemented with appropriate antibiotic and then incubated at 37°C, overnight. This will allow the selection of transformed colonies.

2.2.4 DNA Purification

Single colonies from transformation plates were picked up and added individually to 10ml LB broth in 25ml Falcon tube containing 50µg/ml carbenicillin. Cultures were incubated at 37°C, shaking at 200rpm overnight. Next day, the cultures were centrifuged 4000rpm for 7 minutes to pellet the cells, and the supernatants were discarded. Plasmid DNA was then purified using the QIAprep Spin Miniprep kit according to the manufacturers protocols (QIAGEN). The DNA concentration was then determined by diluting the DNA in MilliQ water at 1:200 and then measuring the absorbance at 260nm in a UV spectrophotometer (Cary UV Vis Spectrophotometer).

DNA purified following mutagenesis or cloning process was run on 1% agarose/TAE gel with 0.5µg/ml ethidium bromide to confirm the DNA purification. Gels were run for 100V for 1hr using a BioRad mini-sub@cell GT gel tank and BioRad power pack 300. Gels were photographed under long wave UV light. DNA concentration was determined using absorbance at 260nm in a UV spectrophotometer. DNA was sent to check for sequence analysis (Geneservice).

2.2.5 Cloning

Fragments of alpha or beta synuclein promoters were cloned into a plasmid reporter system. BAC clones containing the genomic sequences of alpha and

beta synuclein genes were purchased from RZPD (RPI-27M7 and RZPD1.0). This process included three steps, starting with PCR, then ligation and finally transformation, and was used to clone β -Syn (-3742/1) into pGL3basic plasmid vector.

2.2.5.1 PCR Amplification

PCR reactions were set up in 200 μ l thin wall PCR tubes; 1 μ l DNA (100ng/ μ l); 0.6 μ l 25 μ M forward primer; 0.6 μ l 25 μ M reverse primer; 1 μ l 10mM dNTPs; 10 μ l 5X buffer (Prime star buffer); 0.5 μ l Prime star DNA polymerase (TAKARA) and the volume made up to 50 μ l with nuclease-free water. Tubes were tapped to mix, spun briefly, and placed in Thermo Hybaid PCR Sprint thermo cycler. PCR programme was set as follows: 1 cycle: 98°C for 1 minutes; 35 cycles: 98°C for 10 seconds, followed by 68°C for 4 minutes; 1 cycle: 72°C for 12 minutes. Temperature was hold at 4°C. Samples were then digested by restriction enzymes.

2.2.5.2 Restriction enzyme digestion

The digestion reactions were set up for both PCR product (insert) and plasmid vector (pGL3-basic) as follows: 3 μ g of plasmid vector, 5 μ l of 10X buffer and 1 μ l of each restriction enzyme (XhoI, BamHI and Bgl II) in a 1.5ml tube and made up to 50 μ l with nuclease-free water. The mixture was vortexed gently and spun down and then at the appropriate temperature for 2-4 hours or overnight. The digestion product was then gel-purified using the manufacturers protocols (QIAquick Gel Extraction Kit, QIAGEN) (2.2.5.4). Amplified DNA was digested by using $\frac{3}{4}$ of the PCR product volume (35 μ l) with 1 μ l of each restriction enzyme (XhoI /BamHI), 5 μ l of 10x buffer and made up to 50 μ l with nuclease-free water.

2.2.5.3 Dephosphorylation of Vector DNA

The digested DNA vector was dephosphorylated by treatment with shrimp alkaline phosphatase SAP (Roche) to prevent the self-ligation of the plasmid. The mixture of 3µg digested vector and 3µl shrimp alkaline phosphatase with 3µl 10x SAO buffer were incubated at 37°C for 15 minutes. The enzyme was then inactivated at 65°C for 15 minutes.

2.2.5.4 DNA Extraction

This was performed by agarose gel electrophoresis. 2µl of the digested mixtures with 3µl loading dye, were loaded onto 1% agarose gel/TAE with 0.5µg/ml ethidium bromide. Agarose gel was run at 100V, 400mA for 45 minutes with 1Kb DNA ladder. The separated DNA was then visualised using a UV light source to detect DNA bands corresponding to the expected sizes of the products. The total volume of the digested mixtures was run then to extract the DNA. Fragments of interest were cut off using a clean, sharp razor blade. The DNA was extracted from the gel slices using the QIAquick Gel Extraction Kit according to the manufacturer's protocol. In brief, each gel slice was weighed in colourless tube. Three volumes of buffer QG (supplied within the kit) was added to one volume of gel (100mg ~ 100µl) followed by incubation at 50°C for 10min until gel slice was completely dissolved. One volume of isopropanol was then added to the sample, mixed and applied to QIAquick spin column to bind DNA. Several buffer washes were applied before eluting DNA with nuclease free water. The purified DNA was stored at -20°C.

2.2.5.5 Ligation

2µl of each of the DNA vector and DNA insert were run on 1% agarose gel to estimate the concentrations of both DNA samples. 5µl 0.1µg/µl DNA Ladder (GeneRuler™ 1kb DNA Ladder, 250-10,000bp) was run next to fragments on gel.

DNA Ladder was used for DNA sizing and approximate quantification as provided by manufacturer (Fermentas Molecular Biology Tools). The aim was to use 50ng plasmid vector and 150ng of DNA insert (1:3 ratio) in a total reaction volume of 20µl. The ligation reaction was carried out in 1.5ml tube by adding DNA plasmid and DNA insert, 1µl T4DNA ligase (Promega), 2µl 10X ligation buffer and up to 20µl with nuclease-free water. The reaction mix was incubated at 16°C overnight.

2.2.5.6 Transformation

The product from the ligation reaction was then transformed into XL-2 blue or JM109 competent cells as described previously in 2.2.3.

2.2.6 Protein Expression and Purification

2.2.6.1 Transformation

The alpha synuclein open reading frame was previously cloned into pET15b (for His-tag protein) and pET11a (for untagged protein). Plasmid DNA was transformed into BL-21 competent cells as described previously in 2.2.3.

2.2.6.2 Protein Expression

A single colony was picked from a transformation plate and added to 100ml flask tube with 50ml LB broth with 50µg/ml carbenicillin. The culture was grown overnight at 37°C. The next day, the 50mls were added to a litre of LB with 50µg/ml carbenicillin and incubated at 37°C, 200 rpm, until the cells reached an optical density at 600nm of 0.5-0.8. 1ml of 1M IPTG is used to induce protein expression for 3-4 hours. Cells were pelleted by centrifugation at 6000rpm for 10 minutes and pellets were stored at -20°C prior to protein purification. 1ml of culture was taken out before starting the induction with IPTG and another ml of

culture was taken out before pelleting the cells by centrifuging at 6000rpm as un-induced and induced samples, respectively, to check the protein expression later by running the samples on SDS-PAGE gels.

2.2.6.3 Protein Purification

a. Purification of His-tag protein

IMAC column (Immobilized Metal Affinity Chromatography) was used to purify His-tag proteins. It included two steps, starting with preparing the lysate and then loading it onto the column. Cell pellets were resuspended in 2ml buffer (50mM Tris pH7.5, 10mM MgCl₂) per gram of cell pellet. 4µl of 100mM PMSF (Phenylmethyl Sulphonyl Fluoride) per ml lysate was then added, followed by sonicating (4X 1 minute each) at 75% power. Sonication of lysate was performed on ice. After that, 50µg DNase1 per ml lysate was added and samples were incubated shaking at 37°C, 200rpm for 30 minutes to one hour. Finally, 4ml of 8M Urea buffer pH 7.5 per gram of cell pellet was added (48g Urea, 11.6g NaCl, 10.55g Tris-HCl and dH₂O up to 1L pH7.5), followed by sonicating (3X 1 minute each) at 75% power. The lysate was then centrifuged at 12000rpm, 20 minutes, at 4°C. The next step was to filter the lysate through a 0.45µm filter before applying it onto a nickel affinity column, which had been packed with a bed of 5ml chelating sepharose (GE healthcare). The column was first washed with 5 volumes of Milli Q water (pH7.5), 5 volumes of 0.3M nickel sulphate, 5 volumes of MilliQ water pH7 and equilibrated with 5 volumes urea buffer pH7.5. After the filtrate had been loaded, the column was washed with 25ml of 8M urea buffer pH7.5, 150ml of 25mM imidazole in urea buffer, 25ml of 40mM imidazole in urea buffer and the protein was finally eluted with 300mM imidazole in urea buffer. Proteins were then dialysed in chelex-treated MilliQ water pH7.5 prior to use in aggregation assays.

b. Purification of untagged proteins

Ion Exchange Chromatography IEC was used to purify the untagged synuclein proteins. IEC separates proteins based on their net charge which is dependent on the composition of the buffer used. By adjusting the pH or the ionic concentration of the buffer, various protein molecules can be separated. IEC can be either anion or cation exchange chromatography. In anion exchange chromatography, negatively charged ions bind to positively charged resins, while it is the opposite case in cation exchange chromatography. Then, by increasing ionic strength of the elution buffer, the bound molecules will come off the column and will be eluted.

Cell pellets were resuspended in buffer A (50mM Tris, pH8) with 1mM PMSF, 50µg/ml DNase1 and 50µg/ml Lysozyme. The cells were then lysed by sonication (3X 1 minute each) on ice and 1% (w/v) of streptomycin sulphate was added to precipitate ribosomal proteins. The cell lysate was centrifuged at 8000g for 20 minutes at 4°C and the supernatant was retained. 30% (w/v) ammonium sulphate was then added to the supernatant and stirred at 4°C overnight to precipitate the protein. Next day the mixture was centrifuged at 10000g for 20 minutes at 4°C and the cell pellets were retained. At this stage, the cell pellet was resuspended with buffer A and filtered through a 0.22µm filter (Millipore) before it was loaded onto Q-Sepharose Fast Flow column pre-equilibrated with 2CV of buffer A and connected to a fast protein liquid chromatography (FPLC) system (AKTA FPLC) (Amersham Biosciences). The column was then washed with another 2CV of buffer A. Proteins were eluted using a linear gradient of buffer B (50mM Tris, 1M NaCl pH8). It started with 2CV of 0-20% buffer B, followed by a 2CV wash of 20% buffer B. Finally, 2CV of 20-100% buffer B wash (2CV) was used to elute bound proteins. Fractions containing proteins were identified by size analysis using SDS-PAGE gel electrophoresis with Coomassie staining. This method was used to purify untagged Wild Type α -Syn and all its metal binding mutants used in this project.

2.2.6.4 Dialysis of Protein Fractions

The dialysis tubing was prepared as follows: boiled in 2% (w/v) NaHCO_3 with 1mM EDTA for 5 minutes and rinsed in dH_2O . Protein samples were dialysed in the tubing (MW12-14KDa for His-tagged proteins and MW7KDa for untagged proteins, against chelex-treated MilliQ water pH7. 1gram of chelex was added to 5 liters of MilliQ water and stirred for a minimum of 2hours. Then the water was filtered through filtering paper to get rid of the chelex and pH was adjusted to 7 using 1M NaOH or 1M HCl solutions.

2.2.6.5 Determination of Protein Concentration

a. Bradford Assay

Bradford assay was used to determine protein concentration where protein standards of 0 to 1mg/ml Bovine Serum Albumin (BSA) with 0.2mg/ml intervals were prepared using MilliQ water. 10 μl of each of the standards and of the protein samples as well were prepared. Bradford reagent was diluted 5-fold in MilliQ water. 1ml of this diluted reagent was then added to the 10 μl of standards and samples, vortexed and incubated for 5 minutes at 25°C. Then the solutions were transferred to plastic cuvettes and spectrographically analysed at 595nm in a Cary UV Vis Spectrophotometer. From the standards, a calibration curve was calculated and used to determine the protein concentration of the samples.

Also, the same assay was later performed using a micro-plate (96-well plate) using a plate reader (BMG FLUOstar Omega). 2 μl of each of the standards and the samples were taken out and spread into the wells of the 96-well plate. 200 μl of the diluted reagent was then added to each of the samples using a multi-channel pipette and mixed well. Standards and samples were then incubated for 5 minutes at 25°C. The plate was then put into the plate reader which was set up to measure absorbance at 595nm. A calibration curve was drawn automatically in the machine and unknown protein concentration was therefore determined.

b. UV Absorbance

This method was also used to determine the concentration of recombinant protein. Protein samples (100µl) and appropriate buffer (900µl) were transferred to clean quartz cuvettes and mixed properly. The absorbance at 275nm was measured and in a Cary 50 Bio UV-Visible spectrophotometer (Varian), after it was zeroed using the same buffer. Protein concentration was calculated using $C = A_{275} \times \text{dilution} / \text{molar coefficient}$. Where, C: is concentration (M), A_{275} : absorbance at 275nm. The extinction coefficient of the protein was determined based on its amino acid sequence (us.expasy.org/tools/protparam.html).

2.2.7 Polyacrylamide Gel Electrophoresis

12% separating gels were prepared and poured into the gap between ATTO glass plates and allowed to set. A 4% stacking layer was added to the top of the separating gel. For gradient gel preparation, a 5% acrylamide solution and a 20% acrylamide solution were prepared individually in the pouring stand before mixing them together and pouring them into the gap between the ATTO glass plates. A 12-well comb was then set in the stacking layer to create wells to load the samples in. Protein samples for analysis were mixed with 4x loading dye (~10µl) and dH₂O to make a total volume of 20µl. Protein samples were heated at 100°C for 5min and then loaded into the wells along with a protein size marker and the gels were put into a tank containing running buffer. The gels were run for 40min at 35mA and 260V per gel. The electrophoresis was stopped when the dye reached the bottom of the gel. Under denaturing conditions, proteins were separated by molecular weight. Protein fractions were observed by either coomassie blue staining or western blotting using unstained protein ladder.

Densitometry analysis was performed using Image J programme where the same size shape was placed over the bands and the programme measured the pixel density which was then related to control, usually as percentage.

Recipes for acrylamide gels are shown below:

12% acrylamide gel: 2.3ml dH₂O, 2.8ml 30% acrylamide, 2.6ml 1M Tris (pH8.8), 80µl 10% SDS, 10µl TEMED, 50µl 20% APS.

4% stacking gel: 3.6ml dH₂O, 630µl 30% acrylamide, 1.3ml 1M Tris (pH6.8), 100µl 10% SDS, 10µl TEMED, 50µl 20% APS.

4X loading dye: For 10mls: 4ml glycerol, 2ml 1.5M Tris (pH 6.8), 0.4ml 0.5% Bromophenol blue, 0.4g (4% w/v) SDS, 200mM B-mercaptoethanol, dH₂O to 10mls.

Running buffer: For 1L of 10X: 188g Glycine, 30.2g Tris, 100ml 10% SDS and up to 1L with dH₂O.

2.2.8 Coomassie Blue Staining

The gel will be taken out of the plates and soaked in Coomassie stain (for 100ml: 0.25g Coomassie brilliant blue, 45ml methanol, 45ml dH₂O, 10ml acetic acid) for at least 1 hour. The dye will bind nonspecifically to all proteins and any dye that is not bound to the protein will diffuse out of the gel during destaining steps. Gels are destained in a destaining buffer (For 2L: 100ml methanol, 140ml glacial acetic acid and 1760ml dH₂O) and gently rocked until the protein bands were visualised against a clear background.

2.2.9 Western Blotting

Also referred to as immunoblotting, western blotting is a method used to detect specific protein in a given sample. After gel electrophoresis to separate denatured proteins by molecular weight, proteins were then transferred to a PVDF membrane (nitrocellulose membrane) by a semi-dry transfer method. Polyacrylamide gels and blotting paper were soaked in transfer buffer (for 1L: 500ml 1X Running buffer, 200ml methanol and 300ml dH₂O). PVDF membranes were soaked in methanol and placed on the top to pre-soaked blotting paper. The gel was then placed on top of them. Finally, blotting paper was used on top of the previous layers and care was taken to remove any bubbles while placing all the layers on top of each other. The transfer was performed in a Trans-Blot®Semi-Dry transfer machine (Biorad). The transfer was run at 50mA per gel, 25V for 1.5 hour or 33mA, 6V overnight for proteins of cell lysate transfer.

The membrane was then blocked in 5% milk in PBS-0.1% Tween20 at room temperature (RT) for 30 minutes to 1 hour. The membrane was then incubated in primary antibody diluted at 1:2000 in 1% milk in PBS-0.1% Tween20 for 2 hours at RT or at 4°C shaking overnight. Next, the primary antibody solution was removed and the membrane was washed 3 times in PBS-0.1% Tween20 15 minutes first and then 2X of 5 minutes. Horseradish peroxidase (HRP) conjugated secondary antibody diluted at 1:4000 in 1% milk in PBS-0.1% Tween20 was added to the membrane and incubated shaking at RT for 1 hour. The membrane was then washed 3 times, 5 minutes each and treated with ECL PLUS Western Blotting detection kit (Amersham Pharmacia Biotech) for 5 minutes and exposed to film. A compact X-ray processor was used to develop the film. Analysis of expression levels of α -Syn and tubulin were determined. Intensities of detected bands were analysed and compared using densitometry. Values obtained by densitometry analysis of the bands were normalised to tubulin levels.

α -Syn and its disease associated mutants and its N-terminal metal binding mutants were detected using a mouse primary antibody (Zymed Laboratories Inc.) directed against the C-terminus of α -Syn sequence. It was visualized with a secondary anti mouse antibody (Invitrogen). While α -Syn and its C-terminal mutant (Δ 116-129) was detected using sheep primary α -Syn antibody (Abcam) directed against amino acids 11-26 of α -Syn sequences, and was visualized with a secondary anti sheep antibody (Molecular probes).

2.2.10 Isothermal Titration Calorimetry (ITC)

ITC experiments were carried out in a Microcal titration calorimeter (Microcal VP-ITC). Metal solutions were prepared by dissolving the metal in chelex-treated MilliQ water. Protein samples and metal solutions for calorimetric analysis were diluted from concentrated stocks into 250 μ M and 12mM final concentration, respectively. Copper (CuSO₄) was added to the protein prior to ITC experiments at 2:1 ratio (metal: protein). The pH of the working solutions, of both protein and metal, was adjusted to pH7 with 50mM MOPS buffer as final concentration. All reactions and measurements were conducted in an isolated cell chamber at maintained temperature of 25°C. A series of 60x 2 μ l injections of buffered metal solution were titrated into the well chamber containing 1.2ml of buffered protein solution. A time gap of 2 minutes was allowed between injections to allow system temperature to equilibrate between reactants before proceeding to the next injection. A small amount of sample buffer was simultaneously injected into a reference well, which services as a reference temperature for the machine to equilibrate after each injection. The chamber well was washed thoroughly between each run with up to 10 chamber volumes of MilliQ water using a clean syringe. The machine measured any heat changes generated in the experimental well during each injection as the metal reacted with the protein. The machine recorded the total heat change per second over time. Data was then fitted into a

binding isotherm, which represents the heat change per mole of injectant (metal solution) against metal to protein molar ratio. ITC experiments were repeated three times for each condition to ensure reproducibility. Each experimental condition had a blank run with buffer instead of protein in the chamber well. This data was subtracted from the protein run to account for heat dilution, which is the amount of energy released when metal is titrated into the buffer. The data was analysed using Origin 5.0 software from Microcal and fitted into a regression model to predict the number of binding sites on the protein, binding constants (K_a), and change in enthalpies (ΔH) and entropies (ΔS).

2.2.11 Ferrireductase Assay

This assay was performed using recombinant protein or cell lysate from cells over-expressing the protein. 100 μ M recombinant protein or protein solutions of standardised cell lysates were mixed with a final concentration of 100 μ M ferrozine ((3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)-1,2,4-triazine), 100 μ M NADH, 10mM MOPS pH7. Protein had been exposed to 2 equivalents of copper (II) sulphate and allowed to equilibrate. The reaction was started by the addition of iron (III) citrate to a final concentration between 1 μ M and 100 μ M. the reaction was set up in a final volume of 100 μ l. Samples were performed in triplicates and reactions were carried out in 96-well plates. Changes in absorbance were monitored at 562nm in the plate reader (BMG FLUOstar Omega).

2.2.12 Aggregation Assay

A Thioflavin-T based fluorometric assay (ThT assay) was used to investigate synuclein aggregation and fibril formation *in vitro*. ThT assay is based on the ability of the fluorescent dye Thioflavin-T to bind to β -sheet rich amyloid structures and gives fluorescence at excitation and emission maxima of 450nm

and 480nm, respectively. The dye alone or in the presence of low content of β -sheet structures will produce no fluorescence at these wavelengths. A stock solution of buffer (10mM Tris or 10mM MOPS at pH7.4, and 10mM NaP buffer in optimisation experiments) and metals were prepared in advance. Master mixes of dH₂O, metals (up to the required final concentration), protein (up to the final concentration required), buffer and ThT (at 10 μ M final concentration) were added in order into 1.5ml tubes. Master mixes were gently mixed by inverting the tube 4-6 times. Samples were prepared in triplicates or quadruplicates. A 100 μ l of each mix was then aliquoted into 96-well plate and incubated shaking at 37°C, 600rpm. The fluorescence was measured with different time intervals, averaged and then plotted into a sigmoidal curve. Fluorescence measurements were performed using Fluroskan Ascent software version2.4 (with excitation at 444nm and emission at 485nm) and using BMG FLUOstar Omega plate reader.

Data of ThT fluorescence intensities were processed using SigmaPlot software using nonlinear regression. The ThT fluorescence measurements were plotted as a function of time and fitted by a sigmoidal curve described by the following equation using SigmaPlot:

$$F = y_0 + a / (1 + e^{-(x-x_0)/b})$$

Y_0 is minimum fluorescence, a : $\max Y - \min Y$, X_0 : time to 50% maximum fluorescence. The apparent constant rate, k_{app} , for fibrils growth is given by $1/b$. ThT values were normalised by subtracting the background fluorescence of the buffer, then calculating the fluorescence as a percentage to the maximum value in each set of experiments.

2.2.13 Maintenance and Growth of Mammalian Cells

SHSY-5Y (human neuroblastoma) cells were cultured in 50% (v/v) Dulbecco's Modified Eagle media and 50% (v/v) Ham's F12 supplemented with 10% (v/v)

fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin and 2mM glutamine. Growth conditions were maintained at 37°C and 5% CO₂ in a humidified incubator. Routine culture was performed as follows: 1ml trypsin was added per T25 flask after medium was aspirated and incubated at room temperature (RT) for 2-3 minutes with gentle tapping. 4ml medium was used to inactivate trypsin. Cells were counted using the hemocytometer and plated at 3×10^5 in a new T25 with 5ml medium. This procedure was repeated every 5-6 days until cells are confluent with changing the media every 2 days.

2.2.14 Transfection

Transfections were performed using FuGene 6 transfection reagent (Roche) and genejuice transfection reagent. Both transient and stable transfections were performed.

2.2.14.1 Transient Transfection Prior to Luciferase Assay

SHSY-5Y cells were trypsinised and counted using a hemocytometer in order to plate 8×10^4 cells per well in a 24-well plate in 500µl media. Cells were grown overnight at 37°C with 5% CO₂. The next day, cells were transfected with two individual reporter vectors at the same time. The first is the experimental vector, (pGL3basic), containing the insert of interest and a second plasmid that serves as internal control for the transfection (pRL-SV₄₀ or pRL-TK). Either internal control plasmid serves to control transfection efficacy as results were normalised against it. Transfection was performed using 200ng of the pGL3basic plasmid and 10ng of the control plasmid. A master mix of 100µl serum-free media (DMEM containing no FBS, no antibiotics), 200ng DNA and 0.7µl transfecting reagent (FuGene 6) per well, were mixed in a 1.5ml sterile tube by gentle flicking. The mixture was incubated at RT for 15 minutes and then 100µl of the mix was added into each well of the 24-well plate containing 400µl fresh media. Cells were

returned to the incubator and grown at 37°C with 5% CO₂. After 48 hours of incubation, the media was removed and cells were washed using PBS prior to lysing. Cells were harvested using a Passive Lysis Buffer (PLB) provided in the Luciferase Kit (Promega). The buffer was diluted 5 times with dH₂O and then 100µl of the diluted buffer was added to each well. The plates were rocked for 20 minutes on a shaker before storing at -80°C until analysis.

2.2.14.2 Stable Transfection

SHSY-5Y cells were plated in 6-well plates at 50% confluency a day before transfection and were grown at 37°C with 5% CO₂. The next day, cells were transfected as follows: 50µl serum-free media with 3µl transfecting reagent (FuGene 6) were added into a 1.5ml sterile tube and flicked gently and incubated at RT for 5 minutes. For each cell line, 1µg of DNA was then added to the mix and gently flicked again and further incubated for another 15 minutes at RT. After that the transfection mix was added to each well of the 6-well plate and the plate was returned to the incubator. Cells were selected by adding 400µg/ml G418 sulfate (Calbiochem) 24 hours after transfection and maintained at 100µg/ml to keep the selection. Successful transfection was assessed by western blotting to ensure over-expression of the protein was observed.

2.2.15 Luciferase Assay

SHSY-5Y cells were transfected with two individual reporter vectors at the same time. The first is the experimental vector, (pGL3basic), in which the sequence of interest is cloned upstream of the Firefly Luciferase gene. The second is the control vector, Renilla Luciferase, which serves as internal control for the transfection. The activities of both luciferases were detected in the same sample using the Dual-Luciferase Reporter Assay System (Promega). Cells were defrosted shortly prior to the Luciferase assay. 10µl of cell lysate was aliquoted in

each well of a white 96-well plate. Samples were plated in triplicates. Firefly Luciferase activity was measured first by adding (LARII) reagent to generate a stabilized luminescent signal that will be detected and quantified. Then, the reaction was quenched and the Renilla Luciferase reaction was simultaneously initiated by adding (Stop&Glo) reagent to produce a stabilized signal from Renilla Luciferase that will also be detected and quantified. Finally, the activity of the experimental reporter was normalized to the activity of the control reporter to minimize experimental variability caused by differences in cell viability and transfection efficiency.

Chapter Three: Ferrireductase Activity of Alpha Synuclein

3.1 Introduction

A key issue preventing the general understanding of the synucleinopathies is that the function of α -Syn remains unknown. Since 15% of α -Syn has been found to be membrane-bound (Lee, Choi et al. 2002), several different roles of α -Syn in membrane-associated processes have been proposed such as the regulation of synaptic neurotransmitter and dopamine release (Abeliovich, Schmitz et al. 2000). α -Syn was shown to be a metal binding protein, although the precise role for metal binding and whether it has any effect on the protein's function is not known yet.

Metals, such as copper, iron and zinc, have been reported to exist at high concentrations in α -Syn fibrillar deposits, and were proposed to be important factors that cause α -Syn aggregation (Uversky, Li et al. 2001b). Research has focused mainly on copper and, to some extent, iron. Studies of the coordination of copper binding to α -Syn have failed to reach a consensus result. Initial studies showed α -Syn to bind 5 to 10 molecules of copper (Paik, Shin et al. 1999), while a later study showed α -Syn to bind copper at two high affinity binding sites with more copper able to bind at other lower affinity sites (Rasia, Bertoncini et al. 2005). A more recent study found two, independent, non-interacting copper binding sites in the N-terminus of α -Syn (Binolfi, Lamberto et al. 2008). Therefore, there is significant evidence that α -Syn is a copper binding protein, even though the exact number of binding sites and affinities are still controversial. In a normal functioning organism, these metals are essential for various

constituents of the mitochondrial respiratory chain, as well as other parts of the organism (Ohgami, Campagna et al. 2006). Copper and iron metabolism has been shown to be interrelated as copper-dependant oxidases have been shown to be components of cellular uptake pathways of iron in both yeast (Spizzo, Byersdorfer et al. 1997) and mammals (Nittis and Gitlin 2002). Eukaryotes have evolved complex mechanisms to regulate organismal and cellular concentrations of each metal, as a deficiency or an excess in either copper or iron can lead to metabolic collapse or organ toxicity, respectively (Ohgami, Campagna et al. 2006). Both metals exist primarily in the oxidised form in the environment. Therefore, it is an obligate step to reduce these metals and transport them efficiently across the plasma membrane by high affinity transporters. Several metal transporters and reductases, located in different body organs, have been discovered and studied, such as DMT1 and CTR1 found in the plasma membrane of epithelial cells of the intestine (Ohgami, Campagna et al. 2006). Another example is the Steap protein family, which have been shown to function as metalloreductases and to be expressed in different tissues such as pancreas, prostate and brain (Ohgami, Campagna et al. 2006). In general, metalloreductases have been shown to be highly expressed in tissues relevant to metal homeostasis (Ohgami, Campagna et al. 2006; Ghio 2009), and have also been demonstrated to be membrane-bound proteins (Ghio 2009).

Dopamine depletion is considered to be the neurochemical feature that characterises PD; however, reductions in the level of other neurotransmitters in PD patients have also been detected, including noradrenaline, GABA and acetylcholine (Ach). A decline in noradrenaline levels in PD is the most consistently observed (Gesi, Soldani et al. 2000). The depletion in neurotransmitters in dopaminergic terminals, which is an early event in PD pathophysiology, has been proposed to increase susceptibility to neurotoxins. Tyrosine hydroxylase (TH) is an iron-containing rate-limiting enzyme that catalyses the hydroxylation of L-Tyrosine to dihydroxyphenylalanine (DOPA),

which represents an essential step in the biosynthesis of catecholamines (CA) such as dopamine, adrenaline and noradrenaline (Nakashima, Hayashi et al. 2009). Research in this domain has revealed that the decrease in catecholamine levels observed in PD could occur through oxidative or nitrative damage to TH causing a decrease in its catalytic activity (Ara, Przedborski et al. 1998; Kuhn, Aretha et al. 1999). These studies also suggested that inactivation of TH may lead to the formation of insoluble α -Syn fibrils and aggregates that could induce toxicity. This emphasizes the importance of a normal TH function to maintain normal levels of neurotransmitters and therefore emphasizes the importance of iron (ferrous iron) being a critical part of the enzyme and required for its function.

It has been shown that the levels of iron in PD brains undergo a shift in favour of ferric iron (Fe^{3+}) which constitutes α -Syn's substrate in work presented in this chapter. Hence, a defect in α -Syn activity as a ferrireductase could possibly contribute to the previously mentioned iron increase. Moreover, this could also affect the inactivation of the TH as iron is an important part of the enzyme structure, and lead to the dopamine depletion occurring in PD cases.

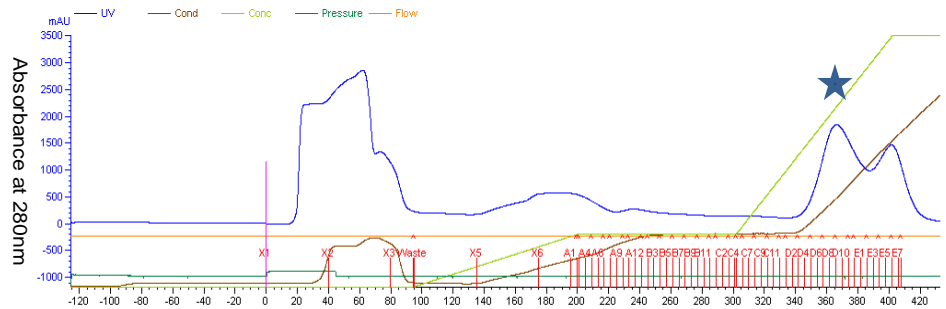
This chapter proposes a possible role of α -Syn as cellular ferrireductase using copper as co-factor and ferric iron (Fe^{3+}) as substrate, in both cell-free (recombinant protein) and cell-based (SHSY-5Y cells) systems.

3.2 Purification of Recombinant α -Syn

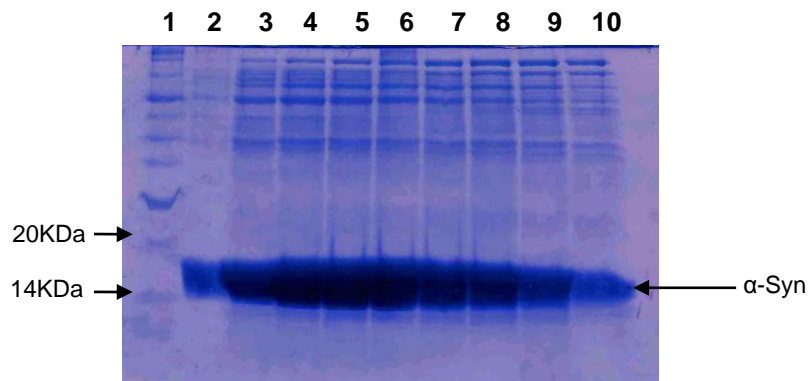
In this study, recombinant His-tagged and recombinant untagged α -Syn was over-expressed and purified. His-tagged protein was used at the early stages of the project to investigate aggregation properties of the protein. After that, untagged protein was over-expressed and purified to further investigate its aggregation properties and ferrireductase activity.

3.2.1 Protein Expression and Purification

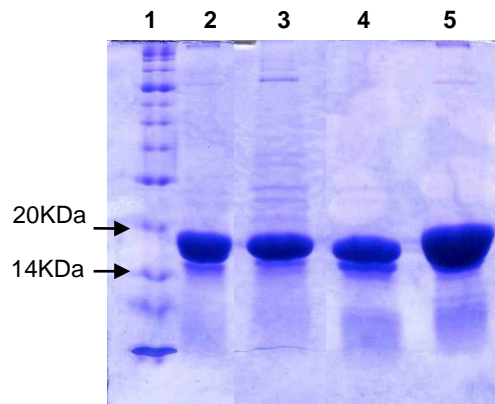
Plasmid vector pET11a encoding untagged α -Syn was transformed into *E.Coli* BL21 expression strain cells. Protein expression was successfully induced with 1mM IPTG. Protein expression was then allowed to proceed for 3-4 hours. Up to 3L of induced cell culture were harvested by centrifugation at 7000g for 7 minutes. The cell pellet was lysed mechanically by sonication (on ice) in buffer A (50mM Tris buffer pH8) with 1mM PMSF, 50 μ g/ml DNase1 and 50 μ g/ml lysozyme. The cell lysate was spun for 20 minutes at 8000g, after adding 1% streptomycin sulphate. Protein in supernatant was precipitated using 30% (w/v) ammonium sulphate stirring over night at 4°C. A second centrifugation step at 10000g for 20 minutes was applied and the resulted pellet was resuspended in buffer A and loaded onto the 50ml anion exchange Q sepharose column pre-equilibrated with 2CV buffer A. The column was then washed with another 2CV of buffer A, followed with a broad gradient wash starting with 2CV of 0-20% buffer B (50mM Tris, 1M NaCl pH8), followed by a 2CV wash of 20% buffer B wash. α -Syn was eluted by 20-100% buffer B wash (2CV). A broad range of low peaks appeared on the UV chromatogram of the purification when applying the low concentration washes of buffer B, while a distinct high peak representing the eluted fractions of α -Syn, appeared during the high concentration flow of buffer B (Figure 3.1, A). Eluted fractions, corresponding to the high peak, containing α -Syn were size analysed by SDS-PAGE gel with coomassie staining (Figure 3.1, B). Same purification method was used for α -Syn metal binding mutants. Fractions containing the protein were pooled and filtered at least two times through a PM30 membrane (Millipore), where most of the high molecular weight contaminants were trapped above the membrane while α -Syn pass through the filter. α -Syn was then concentrated using a PM10 membrane (Figure 3.1, C) and then dialysed against chelex-treated MilliQ water pH7.



(A)



(B)



(C)

Fig. 3.1 Purification of untagged synucleins by anion exchange chromatography. (A) UV chromatogram showing the process of protein loading and elution. (B) α -Syn eluted fractions separated on a 14% SDS-PAGE gel and coomassie staining. Lane1: protein marker. Lanes 2-10: 10 μ l of each eluted fraction. (C) Purified untagged synuclein proteins with anion exchange chromatography. Lane1: Protein marker. Lane2: WT α -Syn. Lane3: H50A α -Syn. Lane4: Δ 1-9 α -Syn. Lane5: Δ 1-9 H50A α -Syn. Star represents the peak at which the protein was eluted.

3.3 Thermodynamics of Iron Binding to α -Syn

Isothermal titration calorimetry (ITC) was used to assess the affinity of iron binding to α -Syn. It is important for this work to show that α -Syn binds iron like it binds copper, as copper is required for the proposed α -Syn ferrireductase activity as a co-factor while ferric iron (Fe^{3+}) is the substrate. Isothermal titration calorimetry (ITC), a calorimetric technique, was used to assess the dynamics of ferric iron (Fe^{3+}) binding to α -Syn. α -Syn was expressed and purified as described previously (2.2.6). The concentration of α -Syn was maintained at 250 μM and titrated with Fe^{3+} 12mM into the chamber of the ITC machine. Experiments were performed by titrating Fe^{3+} into α -Syn in the absence and the presence of copper. In some of the experiments, α -Syn was loaded with two equivalents of copper (CuSO_4) to show that copper-loaded α -Syn is able to bind Fe^{3+} with a similar affinity to monomeric α -Syn in the absence of copper. The use of 2:1 Cu^{2+} : α -Syn stoichiometry was based on recent studies showing that α -Syn is a high affinity copper binding protein at nanomolar range 1:1 stoichiometry (Davies, Wang et al. 2011; Dudzik, Walter et al. 2011). Therefore, 2:1 ratio will enable binding of copper to α -Syn. Both the metal solution present in the injector and the protein sample present in the chamber were buffered using 50mM MOPS pH7 (2.2.10). A background titration was performed where the identical ferric iron (Fe^{3+}) solution was titrated into the chamber that is containing only the buffer (50mM MOPS pH7), as used in the experiments. This background titration was subtracted from each experimental titration, where α -Syn is present, to account for the heat of dilution. The heat of dilution represents the amount of energy released from the occurring dilution when metal is titrated into buffer. The energy change against the molar ratio is indicated by dots in the ITC data plots where the buffer run has already been subtracted (Figure 3.2). The resultant isotherm of the ITC experiments representing the interaction between α -Syn and ferric iron in the absence of copper is shown in Figure 3.2 (a representative isotherm of one experiment). This experiment was repeated three times at 25°C.

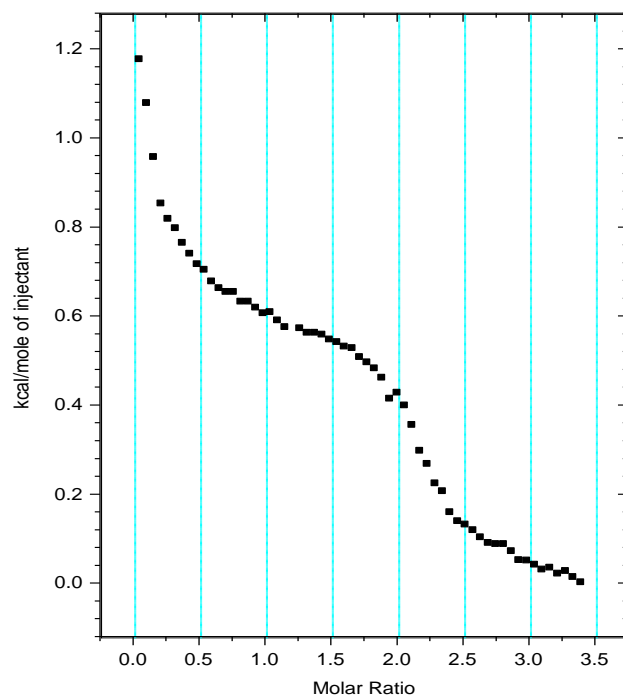


Fig. 3.2 ITC analysis of ferric iron (Fe^{3+}) binding to untagged α -Syn in the absence of copper. The curve represents the heat enthalpy changes after addition of metal as a function of total metal/protein molar ratio in the chamber well.

The titration of Fe^{3+} to α -Syn produced an exothermic response. The data best fit with a two binding sites model using the Microcal Origin 5.0 software, until the least squares regression X^2 value was obtained. Using regression to fit the isotherm of the ITC data, the two binding sites model assumed two sites for Fe^{3+} binding, with different binding affinities and enthalpies, where one of the sites has a higher affinity comparing to the other site. This is also supported by the binding isotherm shown earlier in figure 3.2, where two phases of binding can be observed. The binding constants of the two Fe^{3+} binding sites to α -Syn in the absence of copper are shown in Table 3.1.

A similar result was also obtained when copper-loaded α -Syn was titrated with Fe^{3+} at 25°C, pH7. The background titration was similarly subtracted from each experimental titration, where α -Syn is present, to account for the heat of dilution. The energy change as a function of the molar ratio of Fe^{3+} binding to α -Syn showed considerable binding and the resulted ITC isotherm is shown in the following figure (Figure 3.3) after subtracting the buffer run.

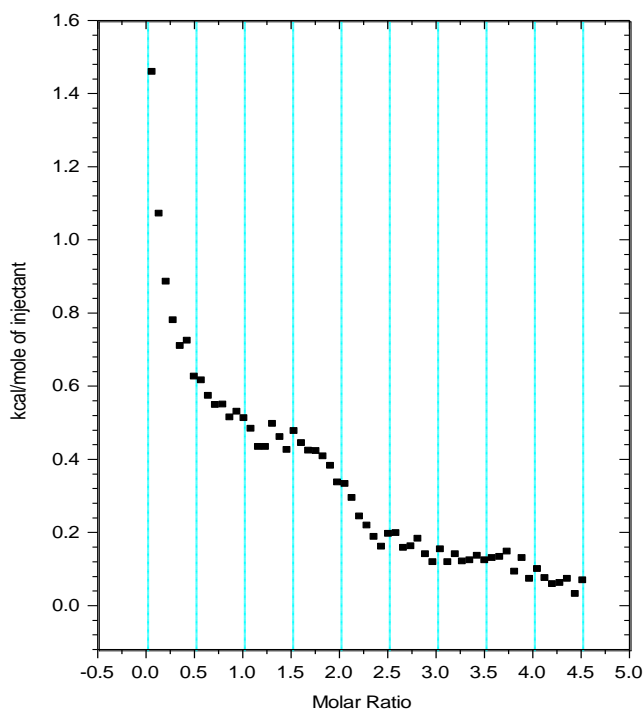


Fig. 3.3 ITC analysis of ferric iron (Fe^{3+}) binding to untagged α -Syn in the presence of Cu^{2+} . The curve represents the heat changes after addition of metal as a function of total metal/protein ration in the chamber well. α -Syn was preloaded with two equivalents of copper (CuSO_4). This figure is of one representative experiment and experiments were repeated three times.

The titration of Fe^{3+} to copper-loaded α -Syn resulted in a similar-pattern of an exothermic reaction. Using Microcal Origin 5.0 software, data was best-fitted with

a two binding sites model until the least square regression X^2 value was obtained. In the presence of copper, the model similarly suggested two binding sites with different affinities and enthalpies, where one has a lower affinity comparing to the other. The ITC isotherm was similar in both cases where copper is present or absent. Table 3.1 shows the stoichiometry and binding constants of Fe^{3+} binding sites to α -Syn in the presence and the absence of copper.

α -Syn+ Fe^{3+}	n1	$K_1 (\text{M}^{-1})$	n2	$K_2 (\text{M}^{-1})$
No Cu^{2+}	0.142767 \pm 0.067	2.13 $\times 10^6$	1.984 \pm 0.055	1.06 $\times 10^5$
With Cu^{2+}	0.182767 \pm 0.088	4.43 $\times 10^5$	1.98 \pm 0.108	3.17 $\times 10^4$

Table 3.1 Comparison of binding constants and number of binding sites of ferric iron to α -Syn in the absence and the presence of copper. Experiments were performed using 250 μM α -Syn and 12mM of Fe^{3+} , when copper was added α -Syn was pre-loaded with 500 μM Cu^{2+} . Each set of experiments were repeated three times. n1 and n2 represent binding stoichiometry and indicate the number of sites detected within each group of sites.

n1 and n2 refer to the stoichiometry of ferric iron binding to α -Syn. The sum of N values for different sets of sites indicated the number of sites. It shows that α -Syn, in the presence or the absence of copper, binds two ferric iron molecules with an error of less than 10%. K values, which represent the binding constants, were calculated from the mean of three independent experiments with errors also less than 10%. The data suggested two binding sites of Fe^{3+} where one of them has lower affinity comparing to the other site. ITC also provided association constants values (K_a) from which dissociation constants values (K_d) can be obtained which represents the measure of how tightly a ligand binds to a particular protein (Table 3.2).

α -Syn+Fe ³⁺	K ₁ (M ⁻¹)	K _{d1} (μ M)	K ₂ (M ⁻¹)	K _{d2} (μ M)
No Cu ²⁺	2.13x10 ⁶	0.469	1.06x10 ⁵	9.433
With Cu ²⁺	4.43x10 ⁵	2.257	3.17x10 ⁴	31.545

Table 3.2 Calculations of dissociation constant values of WT α -Syn from Fe³⁺ in the presence and absence of copper, based on affinity constant values obtained from ITC data.

Enthalpy changes (ΔH) as calculated by ITC binding experiments showed that ΔH values of the binding events of Fe³⁺ to α -Syn were relatively close, thus reflecting sites with similar level of energy capacities. In addition to that, assessment of the free energy changes in the binding reaction showed the following results (Table 3.3), where the Gibbs free energy states of the iron binding process would favour a forward reaction at physiological concentrations and conditions.

α Syn+Fe ³⁺	n1	K ₁ (M ⁻¹)	ΔG_1 (KJ)	n2	K ₂ (M ⁻¹)	ΔG_2 (KJ)
No Cu ²⁺	0.142767	2.13x10 ⁶	-7899.37	1.984	1.06x10 ⁵	-7067.24
With Cu ²⁺	0.182767	4.43x10 ⁵	-7618.85	1.98	3.17x10 ⁴	-6245.33

Table 3.3 Free energy changes of the binding events as shown by ITC experiments

3.4 Ferrireductase Activity of α -Syn

One way to assess whether α -Syn displays ferrireductase activity is to use recombinant protein. A ferrozine-based colorimetric assay, known for its potential to detect and even quantify iron, was used to assess α -Syn ferrireductase activity (Riemer, Hoepken et al. 2004). Ferrozine (3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)-1,2,4-triazine) forms a complex with ferrous iron (Fe²⁺) that strongly

absorbs light at 562nm. The first experiment was conducted to obtain a standard plot (calibration curve) of ferrous iron (Fe^{2+}) concentrations against absorbance at 562nm. This calibration curve was used for the determination of ferrous iron (Fe^{3+}) concentrations. A range of standard concentrations of equal volumes of Fe^{2+} (used as sulphate form), was added anaerobically and mixed with 100 μl ferrozine solution prepared at 100 μM final concentration. After applying the ferrozine solution into ferrous iron (Fe^{2+}) standards, the absorbance at 562nm was measured and it was found to rapidly increase to maximal values within 10 minutes and produce a linear plot (Figure 3.4).

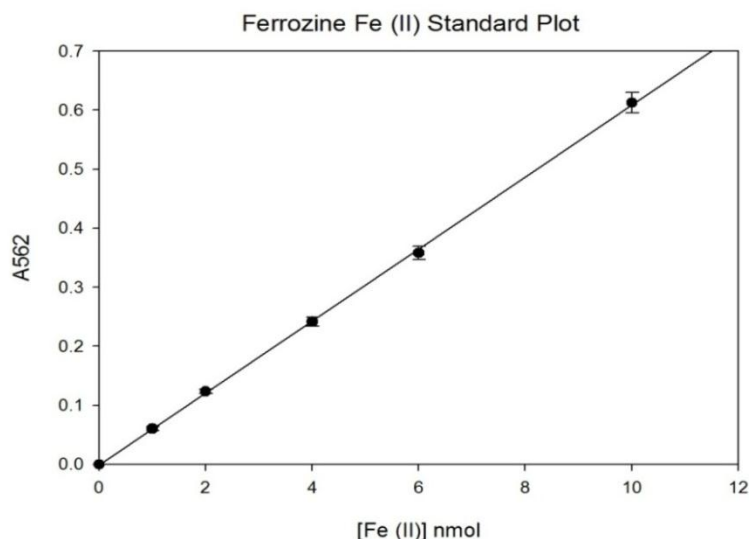


Fig. 3.4 Absorbance of the Fe^{2+} -ferrozine complex formed with increasing concentrations of the standard (ferrous sulphate). The amount of iron indicated was contained in 100 μl in wells of a 96-well plate. The data represent means \pm SD of triplicates of a representative experiment.

3.4.1 Determination of α -Syn Ferrireductase Activity

For the assessment of ferrireductase activity purified recombinant protein WT α -Syn was used at 100 μM final concentration. The ferrireductase activity of α -Syn was stimulated by the addition of 100 μM NADH as an electron donor and in the

presence of copper (Cu^{2+}) as a possible co-factor. Studies have shown that α -Syn is a copper binding protein, however it is still controversial to how many copper molecules can α -Syn bind. Therefore, a ratio of 2:1 copper: α -Syn was used in ferrireductase assays described in this chapter to ensure that α -Syn is present in the copper-bound form. Controls in the absence of copper, protein or NADH were used to ensure any observed activity is due to the copper-loaded protein.

The assay was carried out in 96-well plates at a total volume of 100 μ l per well. Samples representing each condition were performed in triplicate. 100 μ M α -Syn was loaded with 2-fold copper in the sulphate form. Protein solutions were buffered with 10mM MOPS, pH7. 100 μ M of each of NADH and ferrozine were added to the buffered copper-loaded protein. The final step was then to add the ferric iron (Fe^{3+}), and start measuring and monitoring changes in the absorbance at 562nm in BMG Fluorstar plate reader.

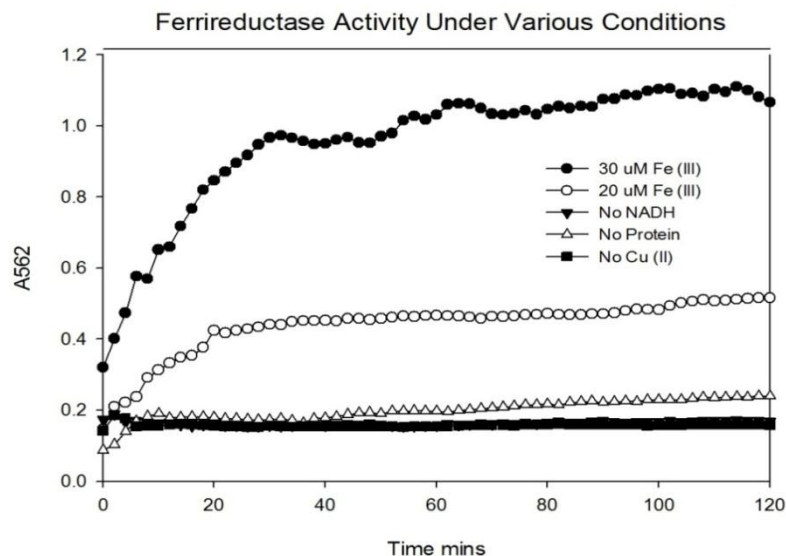


Fig. 3.5 Changes in the absorbance of Fe^{2+} -ferrozine complex at 562nm in the presence of α -Syn and two different ferric iron concentrations. The assay was performed in 96-well plate with 100 μM α -Syn loaded with 2-fold copper, 100 μM NADH, 100 μM ferrozine and either 20 μM or 30 μM Fe^{3+} in the citrate form. The bottom three lines (white triangles, black triangles and black squares) represent controls used in the experiment (without protein, NADH or copper respectively).

Ferric iron (Fe^{3+}) at concentrations of 20 μM and 30 μM was reduced by copper-loaded α -Syn in the presence of NADH. This is clear from the increase in the absorbance at 562nm, which represents an increase in the formation of Fe^{2+} -ferrozine complex. This catalytic activity of α -Syn was shown to be dependent on the protein being loaded with copper (Cu^{2+}), as no increase on absorbance at 562nm was observed when copper is absent in one of the controls (Figure 3.5). In addition, increasing the concentration of the substrate resulted in an increase in the rate of the reaction. This is further illustrated in the following figure (Figure 3.6).

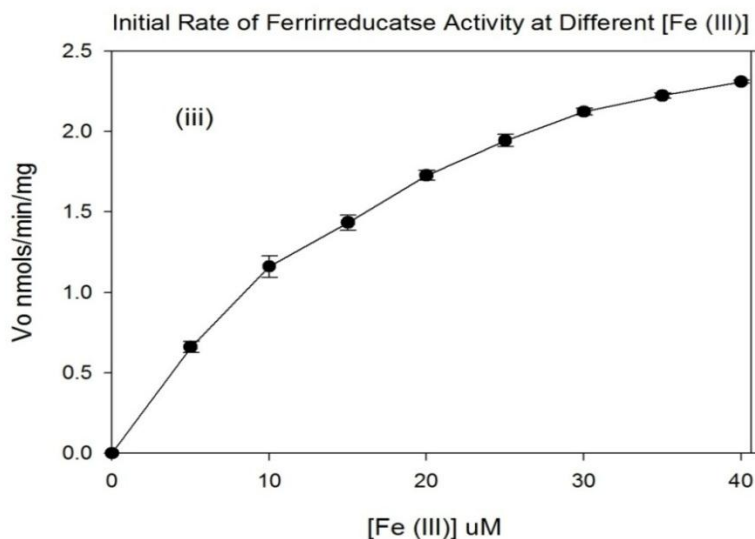


Fig. 3.6 Initial rates of activity based on substrate concentration. Increasing Fe^{3+} concentration resulted in an increase in reaction velocity to reach a maximum at around $50\mu\text{M}$ Fe^{3+} . The data represent means $\pm\text{SD}$ of triplicate of a representative experiment.

The rate of α -Syn activity in reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is dependent on the substrate concentration. It is shown from figure 3.5 that the reaction is following first-order kinetics, because the reaction rate is directly proportional to substrate concentration. Therefore, the use of a range of increased substrate (Fe^{3+}) concentrations from $1\mu\text{M}$ to $50\mu\text{M}$ led to an increase in the reduction reaction until the velocity of the reaction reached a maximum. The maximum velocity represents the stage at which the substrate concentration is high enough to saturate all available enzyme molecules. However, in this case of α -Syn, it is more common to measure the substrate concentration at which the reaction rate reaches half of its maximum value. This concentration is expressed as the K_m , which describes the relationship between the reaction rate and the substrate concentration for a particular catalytic molecule (enzyme).

A more accurate determination may be made through a double-reciprocal plot which yields a straight line, based on the Michaelis-Menten equation of enzyme kinetics as shown in Figure 3.7. V_{\max} is difficult to be determined from plot in Figure 3.6, since enzymes may not function optimally in the presence of excessive amounts of substrate and V_{\max} may not be achieved in enzymatic reactions.

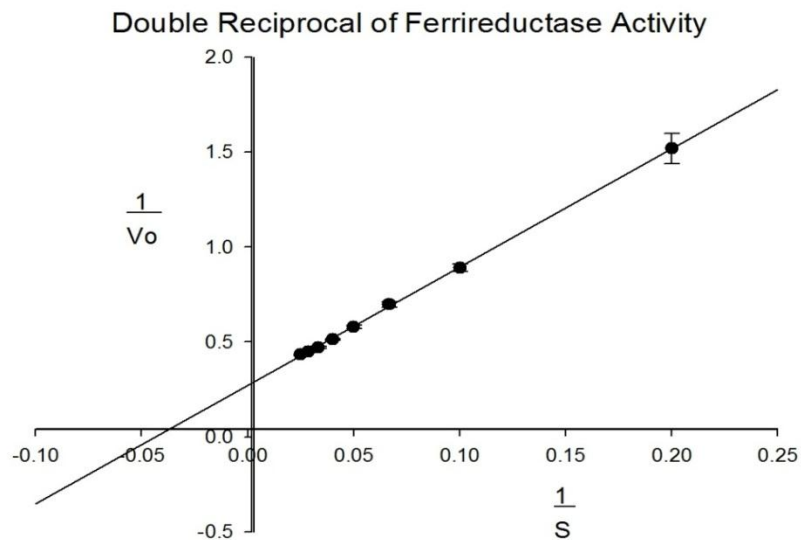


Fig. 3.7 Double-reciprocal plot for the calculation of V_{\max} and K_m . V_{\max} is the reciprocal of the Y intercept of the straight line. K_m is the negative reciprocal of the X intercept of the same line.

The double-reciprocal plot of V_0 against $[S]$ yields a straight line. The reciprocal was taken for both substrate concentration and the velocity of α -Syn reduction activity. From these plots, the V_{\max} and K_m were calculated for the reaction of the reduction of ferric iron to ferrous iron mediated by α -Syn, where V_{\max} (2.72 nmol/min/mg) is the reciprocal of the Y intercept of the straight line. K_m (23 μ M) is the negative reciprocal of the X intercept of the same line (Table 3.4).

3.4.2 Determination of Ferrireductase Activity of Disease-Associated Mutants

Disease-associated mutants of α -Syn (A30P, A53T, E46K) were assessed for ferrireductase activity using the same procedure used with WT α -Syn. Recombinant disease-mutant proteins were purified and used at final concentration of 100 μ M in the ferrireductase assay. The assay was carried out with copper-loaded protein, buffered to pH7 with 10mM MOPS. 100 μ M NADH and 100 μ M ferrozine were then added to the protein solution. Adding ferric iron was the last step before measuring changes in absorbance at 562nm due to the formation of Fe^{2+} -ferrozine complex (Figure 3.8).

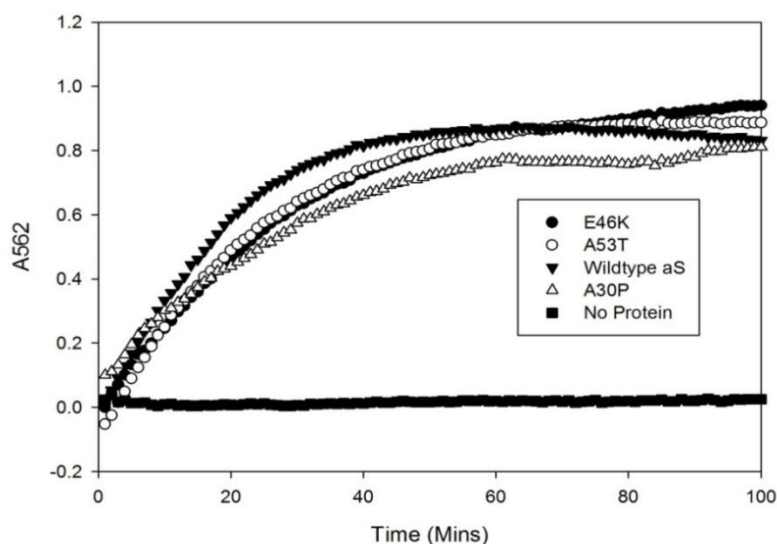
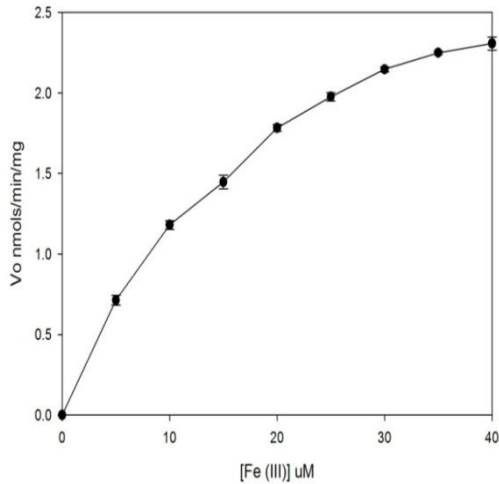


Fig. 3.8 Changes in the absorbance of Fe^{2+} -ferrozine complex at 562nm in the presence of 100 μ M Fe^{3+} . The assay was carried out in 96-well plate using 100 μ M final protein concentration loaded with 2-fold copper, with 100 μ M NADH, 100 μ M ferrozine.

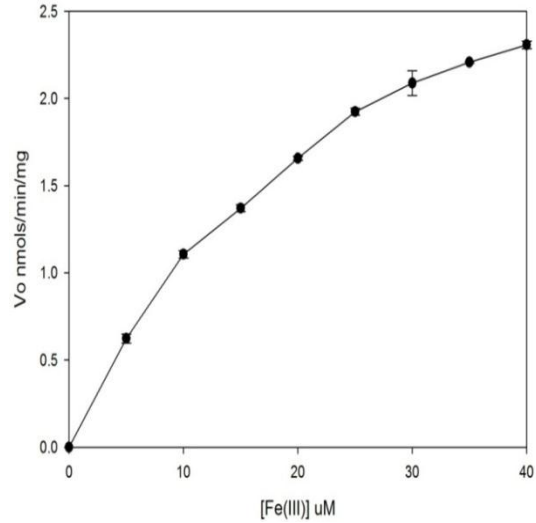
The disease mutants (A30P, A53T, E46K) share similar catalytic activity of WT α -Syn in reducing ferric iron into ferrous iron, and this activity is also dependent on

the protein being loaded with copper. The absorbance increased with increasing substrate (Fe^{3+}) concentrations, which also means an increase the rate of the reaction. Using a gradient in substrate concentrations from $5\mu\text{M}$ and up to $40\mu\text{M}$ led to increase in rate of the reaction for each of the mutant proteins (Figure 3.9).

Initial Rate of Ferrireductase Activity at Different $[\text{Fe (III)}]$ for A30P

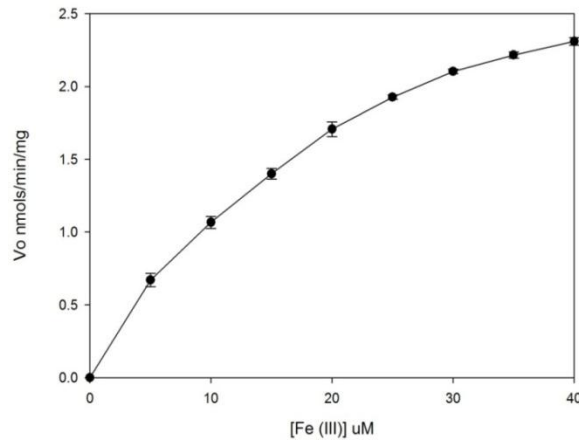


(A)



(B)

Initial Rate of Ferrireductase Activity at Different $[\text{Fe (III)}]$ for E46K



(C)

Fig. 3.9 Initial rates of activity of the disease mutants of α -Syn in relation to substrate concentration. Increasing Fe^{3+} concentrations resulted in an increase in the reaction velocity up till $50\mu\text{M}$ Fe^{3+} in the presence of A) A30P α -Syn, B) A53T α -Syn and C) E46K α -Syn at $100\mu\text{M}$ final concentration. The data represent means $\pm\text{SD}$ of triplicate of a representative experiment for each of the mutant proteins.

The rate of activity of the synuclein disease mutants in reducing Fe^{3+} into Fe^{2+} was dependent on the substrate concentration as shown in figure 3.9. When using a gradient of increased Fe^{3+} concentrations, the resultant rate was accordingly increasing. To better determine the velocity of the reaction and the K_m , the data were plotted into a double-reciprocal plot, which gives a straight line from which a more accurate V_{\max} and K_m values can be obtained (Figure 3.10).

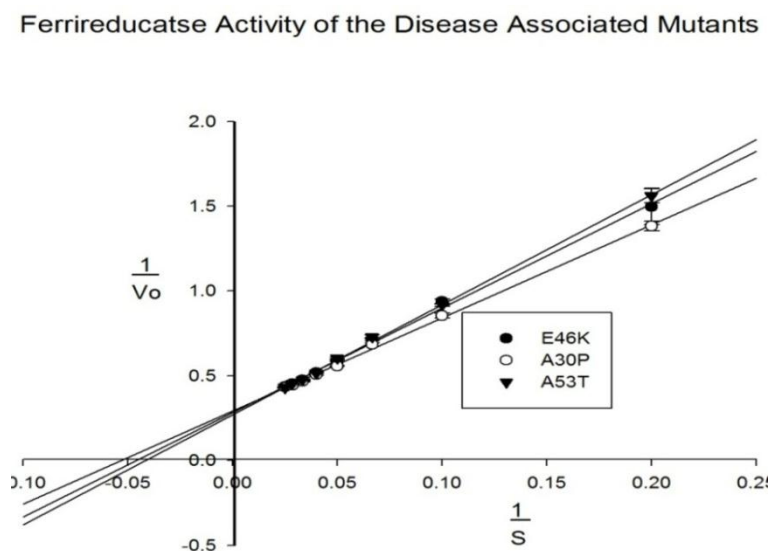


Fig. 3.10 Double-reciprocal plot for the calculation of V_{\max} and K_m . V_{\max} is the reciprocal of the Y intercept of the straight line. K_m is the negative reciprocal of the X intercept of the same line.

Values for V_{\max} and K_m were calculated and listed in table 3.4 for comparison with WT α -Syn.

Protein	V _{max} (nmols/min/mg)	K _m (μM)
Wild Type α-Syn	2.72	23
E46K α-Syn	2.52	23
A30P α-Syn	2.37	19
A53T α-Syn	2.62	25

Table 3.4 Comparison of the kinetic parameters for the ferrireductase activity of wildtype α-Syn and its disease-associated mutants showing the calculated values for each of Vmax and Km as described above.

3.5 Discussion

α-Syn fibrils have been recognised as hallmarks in brains of PD and other neurodegenerative diseases (Spillantini, Crowther et al. 1998b). Transition metals, such as copper and iron, have also attracted attention in the research of neurodegenerative diseases. Both metals have been found to accelerate α-Syn aggregation and to bind to it, where α-Syn is widely accepted as copper binding protein (Uversky, Li et al. 2001b; Binolfi, Rasia et al. 2006). Considering that one of the main unknown aspects about α-Syn is its function and whether metal binding to α-Syn contribute in one way or another to its function; work in this chapter represents an attempt to explore a possible aspect of α-Syn's function as ferric-reductase and hence link this function to its ability to bind metals including iron. Studies of metal binding to α-Syn have centred more on copper than iron, although the exact features of that binding is not established yet. There has been some degree of variability in the proposed number of copper binding sites and their affinities. Up to 10 molecules of copper were suggested to bind to one molecule of α-Syn (Paik, Shin et al. 1999), while in later studies α-Syn was shown to bind two molecules of copper (Rasia, Bertoncini et al. 2005; Binolfi, Lamberto et al. 2008). However, based on recent work conducted in our laboratory, α-Syn was shown to be able to bind one equivalent of copper.

Although the number of copper molecules that bind to α -Syn is controversial, accumulative evidence has shown α -Syn as copper binding protein and this is important as α -Syn's ability to reduce ferric iron (Fe^{3+}) occurred only when copper was present (Figure 3.4). This implies that copper might be playing a co-factor role in the reaction. It is important to note that two equivalents of copper were used in ITC experiments as copper at higher concentrations caused instant α -Syn aggregation and precipitation in the sample while repeating experiments. However, it was more crucial for this project to assess and demonstrate that ferric iron binds to α -Syn considering that it is the substrate in the reaction. Notably, the C-terminus of α -Syn has been referred to as a possible site to bind iron and other metals (Rasia, Bertoncini et al. 2005; Binolfi, Lamberto et al. 2008), although the features of the iron binding are not yet studied thoroughly. Furthermore, it was equally important to show that α -Syn binds ferric iron in the presence of copper as both metals are part of the reaction.

ITC is a method commonly used to study ligand-binding properties to proteins based on the assessment of the heat changes during the reaction. ITC was used to reveal binding properties of Fe^{3+} to α -Syn, as Fe^{3+} resembles the substrate in the reaction described in this chapter. ITC produces curves that reflect heat changes within the binding reaction and the shape of the curves is dependent on the binding mode. A previous study that has attempted to reveal Fe^{3+} binding properties to α -Syn (Bharathi and Rao 2007) has suggested a single Fe^{3+} binding site on α -Syn, utilising the same technique which is ITC. However, work done in this study suggested two binding sites of Fe^{3+} on α -Syn as ITC isotherms were best fitted with a two binding sites model and reflected two binding phases as well (Figure 3.2 and Figure 3.3). The affinity values (K_a) of the two binding groups (n_1 , n_2), in the presence or the absence of copper lay within the same range. This suggests that the presence of copper did not change the binding affinity of ferric iron to α -Syn and reserved the main characteristics of iron binding. Therefore, α -Syn binds ferric iron (Fe^{3+}) at two sites, with relatively similar affinity

values (K_a). These binding affinity values (K_a) represent the association constant values which describes the affinity between Fe^{3+} and $\alpha\text{-Syn}$ at equilibrium. K_a is the inverse of the dissociation constant value (K_d), which is usually the propensity of a complex molecule to dissociate to its components. In other words, it is the concentration at which half Fe^{3+} molecules are not bound to $\alpha\text{-Syn}$. This value is of a similar magnitude to the K_m value obtained for $\alpha\text{-Syn}$ as a ferrireductase. K_m describes the substrate concentration where the reaction of a certain catalytic molecule reaches half the maximum rate. K_d values were calculated for $\alpha\text{-Syn}$ based on K_a values obtained from ITC performed with and without copper as shown in table 3.2. It shows that, when $\alpha\text{-Syn}$ is loaded with copper, the affinity for Fe^{3+} decreases slightly in both sites. However, the main aspect is that the dissociation constant value (K_d) when $\alpha\text{-Syn}$ is pre-loaded with copper is comparable and close to the K_m value ($23\mu\text{M}$) for $\alpha\text{-Syn}$ (Table 3.4). Also, the conditions used in ITC experiments were similar to the conditions in which ferrireductase assays were performed, therefore, the comparison between K_d values and K_m values is valid in such case.

Moreover, assessment of the ferrireductase activity of $\alpha\text{-Syn}$ using the ferrozine-based assay showed that $\alpha\text{-Syn}$ activity follows first order kinetics, where the rate of the reaction was directly proportional to substrate concentration as shown in Figure 3.4. Figure 3.11 illustrates the classical Michaelis-menten enzyme kinetics where K_m is the amount of substrate needed to reach half maximum velocity at which half substrate is bound to enzyme. In other words, K_m is another representative of K_d (the dissociation constant) for $\alpha\text{-Syn}$. K_m values for the reaction between Fe^{3+} and $\alpha\text{-Syn}$ was calculated from the linear plot in Figure 3.7 and Figure 3.10 and presented in Table 3.4.

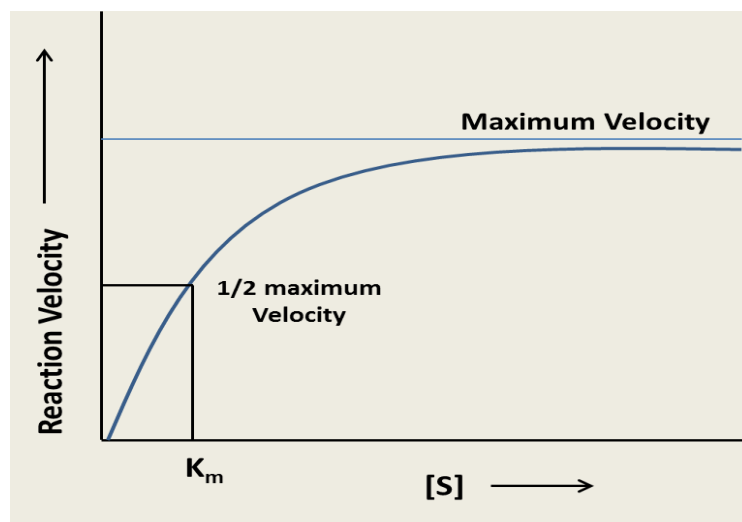


Fig. 3.11 The curve of velocity versus substrate concentration as derived from the Michaelis-Menten theory of enzymatic activity.

The difference between K_d values obtained from ITC binding studies and K_m values obtained from ferrozine-based assay for α -Syn are relatively small, providing evidence that the process of iron binding pre-reduction is the same process as that observed in the ITC assays. Therefore, it is possible to conclude that both methods confirm the proposed ability of α -Syn to bind and reduce ferric iron (Fe^{3+}) in the presence of copper as co-factor.

In terms of free energy changes, Table 3.3 showed that ΔG values did not change between the two binding sites and the presence of copper did not affect the change of energy pattern dramatically. ΔG value is lower for one of the binding sites comparing to the other site, which suggests that the binding is possible from energy point of view and is favorable. Fe^{3+} could bind to α -Syn on the first binding site (site1) and then move to the second binding site (site2) where copper is the redox centre and mediate the ferric iron (Fe^{3+}) reduction into ferrous iron (Fe^{2+}) (Figure 3.12).

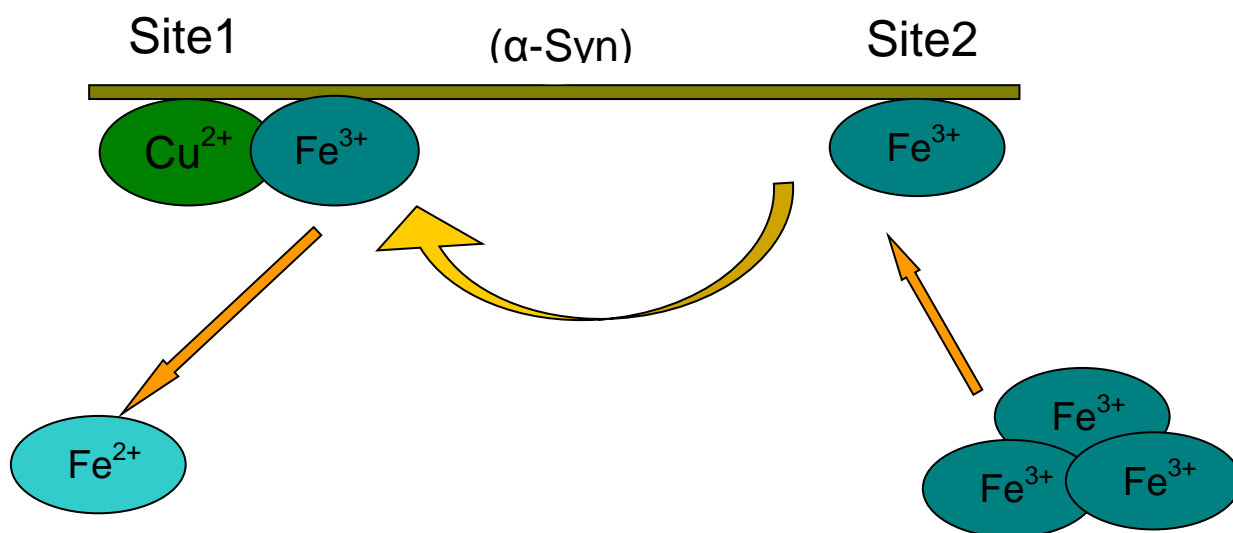


Fig. 3.12 Theoretical pathway of ferric iron reduction by α -Syn in the presence of copper as co-factor. Fe^{3+} binds to α -Syn at the first binding site with $\Delta G = -6245.33$ and then moves to the second binding site with $\Delta G = -7618.85$ which is energetically favourable.

The figure above represents a speculation pathway in which α -Syn reduces Fe^{3+} to Fe^{2+} using Cu^{2+} as co-factor and NADH as electron donor, based on data obtained from ITC and ferrireductase experiments performed with recombinant α -Syn. In this model and in the presence of copper, α -Syn can bind two ferric iron (Fe^{3+}) molecules, and reduce them in an energetically favourable route. The reduction of ferric iron to ferrous iron has been shown to be an important process in the uptake of iron in many organisms such as *Saccharomyces cerevisiae* (Inman, Coughlan et al. 1994; Lesuisse, Casteras-Simon et al. 1996; McKie, Barrow et al. 2001). This process of iron uptake to many cell types has been suggested to be mediated by transferrin (ferric iron carrier) and Dmt1 (ferrous iron selective carrier) (Ohgami, Campagna et al. 2005) where the presence of an NAD(P)H-dependent, cell-surface and/or endosomal ferrireductase activity is required (Ohgami, Campagna et al. 2005). This supports the proposed role of α -Syn as ferrireductase, since it is a membrane bound protein and NADH was required for its activity. In addition, this further confirms the importance of balanced metal

content in the brain, which is altered in disease status and could be attributed to a change in α -Syn structure or function.

Chapter four: Aggregation of Alpha Synuclein

4.1 Introduction

Considerable evidence suggests that the aggregation of α -Syn represents a critical step in the aetiology of PD (Trojanowski and Lee 2003). This is supported by various observations such as over-expression of α -Syn in familial PD cases with multiplications in α -Syn gene (SNCA) (Kruger, Kuhn et al. 1998; Zarranz, Alegre et al. 2004). In some cases of early onset familial PD, the cause is linked to duplication or triplication in the α -Syn gene locus (Singleton, Farrer et al. 2003). Additionally, α -Syn disease associated mutants (A30P, A53T) were shown to increase the propensity of α -Syn to aggregate (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998; Zarranz, Alegre et al. 2004). More importantly, α -Syn fibrils are found within cells in the form of neuronal cytoplasmic inclusions (LBs and LNs) in the brains of PD patients (Jakes, Spillantini et al. 1994).

α -Syn is known to be a natively unfolded protein, although it can adopt other structural conformations, such as α -helical in its lipid-bound state (Clayton and George 1998; Chandra, Chen et al. 2003). In the fibril form α -Syn has been shown to adopt a cross- β structure of unknown function (Conway, Harper et al. 2000; Serpell, Berriman et al. 2000). It is essential to highlight this structural transformation of α -Syn into β -sheet rich fibrils that is found in LBs (Serpell, Berriman et al. 2000), and the possible role of α -Syn aggregates in the specific loss of dopaminergic neurons in PD brains (Winner, Jappelli et al. 2011). This conformational change to β -sheet structure has been previously noted for the A β -peptide of Alzheimer's disease (Barrow and Zagorski 1991) and prion protein

(Pan, Baldwin et al. 1993). This structural change is believed to be an essential step in the pathogenesis of neurodegenerative amyloid diseases. Several factors and conditions have been identified to promote α -Syn aggregation, fibril formation and the likelihood of inclusion formation. These factors include mass crowding (Uversky, Cooper et al. 2002), point mutations (Li, Uversky et al. 2001), the level of phosphorylation (Chen and Feany 2005), and the presence of metals (Paik, Shin et al. 1999; Uversky, Li et al. 2001b). It has been demonstrated that fibrils of recombinant α -Syn formed *in vitro* resemble those derived from disease affected brains (Conway, Harper et al. 1998; Hashimoto, Hsu et al. 1998; Giasson, Uryu et al. 1999).

Research of α -Syn and neurodegeneration has revealed that α -Syn structural change can also lead to aggregated forms other than fibrils, such as amorphous aggregates and oligomers (Uversky, Li et al. 2001a; Bharathi and Rao 2007). However, since fibrillar α -Syn is seen in the surviving neurons in the brain, this has prompted a lot of research into revealing the reasons behind α -Syn transformation into an abnormal form and the consequences of this aggregation. α -Syn fibrils have been reported to have typical morphology to amyloid fibrils with β -sheet structure (Conway, Harper et al. 2000). Several methods have been used to assess and detect this fibrillar protein structure such as Circular Dichorism (CD), electron microscopy (EM) and more simply Thioflavin-T (ThT) fluorescence (Uversky, Lee et al. 2001; Rasia, Bertoncini et al. 2005; Binolfi, Rasia et al. 2006). ThT is a fluorescent dye that has the ability to bind to β -sheet rich amyloid fibrils and gives fluorescence at excitation and emission maxima of 450nm and 480nm, respectively. The dye alone or in the presence of proteins with low β -sheet content gives no fluorescence at these wavelengths. α -Syn aggregation kinetics exhibit sigmoidal curves, defined by an initial lag phase, followed by exponential phase (growth phase) and final plateau after fibrils are completely formed (Uversky, Li et al. 2001a).

Metals have been identified as epidemiological risk factors for PD (Gorell, Johnson et al. 1999) and found to accelerate the fibrillation rate of α -Syn *in vitro* as assessed by laddering on SDS-PAGE gel (Paik, Shin et al. 1999) and by Thioflavin-T (ThT) assay (Uversky, Li et al. 2001b). However, research on the interaction between α -Syn and metals has reported variable information about how metals influence the aggregation process of α -Syn. Several studies have suggested metal binding to α -Syn, thus inducing its aggregation (Paik, Shin et al. 1999; Rasia, Bertoncini et al. 2005; Binolfi, Rasia et al. 2006). The N-terminal domain of α -Syn (residues 1-9 and residues 48-52) including His50 as anchoring residue, as well as residues within the C-terminus (121-123) have been suggested as primarily metal binding sites for metals such as copper and iron. Given that α -Syn has been shown to be a copper and iron binding protein (Bharathi and Rao 2007; Binolfi, Lamberto et al. 2008), metal binding could be the mechanism by which metals induce α -Syn aggregation. Moreover, metals such as copper and iron (redox-active metals) were proposed to play a role in the aggregation of α -Syn via catalysis of oxidative oligomerization in the presence of hydrogen peroxide leading to neurodegeneration (Hashimoto, Hsu et al. 1999; Paik, Shin et al. 2000). This is supported by observations of disturbed metal homeostasis such as increased iron levels and the presence of iron deposits in PD brains (Castellani, Siedlak et al. 2000; Sayre, Moreira et al. 2005). In addition, elevated levels of copper have been reported in the cerebrospinal fluid of PD patients (Pall, Williams et al. 1987). Also, levels of hydrogen peroxide have been shown to be increased with the stimulation of dopamine turnover by monoamine oxidase (Spina and Cohen 1989; Paik, Shin et al. 2000). Redox-active metals and hydrogen peroxide, hydroxyl radicals could be generated in PD and lead to neurodegeneration by affecting various biomolecules including brain proteins (such as α -Syn) and thus contributing to their aggregation (Paik, Shin et al. 2000).

Along with the broad assessment of α -Syn aggregation *in vitro* using recombinant protein, cell culture and animal models have also been used to better understand the mechanism of aggregation and its consequences. This has proved important especially with the accumulating evidence that fibrils may not be the pathogenic species in PD. However, many studies have shown that α -Syn is toxic to neurons in an oligomeric or aggregated form (Dedmon, Christodoulou et al. 2005; Danzer, Haasen et al. 2007; Wright, Wang et al. 2009). The presence of highly soluble oligomers of α -Syn has been reported in cell culture, animal models and in brains with PD and DLB (Volles and Lansbury 2002; Sharon, Bar-Joseph et al. 2003). These oligomeric species of α -Syn have been shown to be able to form pores in the membrane (Volles and Lansbury 2002), which may alter the balance of ions within cells and trigger apoptosis. In addition, linking that to metals, injecting iron to the substantia nigra of adult rats has been shown to cause dopaminergic neurodegeneration in PD (Youdim, Ben-Shachar et al. 1991). Copper has also been shown to participate in the extracellular toxicity of α -Syn due to the formation of a unique oligomeric species (Wright, Wang et al. 2009).

The following work presents an attempt to examine whether α -Syn aggregation is mediated by metal binding. With the recognition of potential sites for metal binding in α -Syn, mutants lacking amino acids proposed to comprise the metal binding sites were created. Both WT α -Syn and its metal-binding mutants were used as recombinant proteins in ThT assays to detect any differences in aggregation kinetics in response to metal treatment. Recombinant α -Syn was purified initially as His-tagged and then untagged protein. To support the assessment of α -Syn metal-binding induced aggregation with recombinant protein, α -Syn and its metal-binding mutants were expressed in neuroblastoma cell line SHSY-5Y and treated with metals, and the extent of aggregation was analysed using western blotting.

4.2 Purification of Recombinant Alpha Synuclein

In this study, recombinant His-tagged and recombinant untagged α -Syn was over-expressed in bacteria and purified by chromatographic techniques. His-tagged protein was used at early stages of the work to investigate general aggregation properties of the protein. The use of His-tagged protein was due to the availability of the purification protocol of His-tagged and not untagged protein. After that, untagged protein was over-expressed and purified, to further investigate its aggregation properties.

4.2.1 Expression and Purification of His-tagged α -Syn

Plasmid vector pET15b- α -Syn was transformed into BL21 cells and α -Syn expression was induced with 1mM IPTG. Due to the presence of the Histidine tag (His-tag), the protein was purified using nickel affinity column (IMAC column). The column was first washed with MilliQ water pH7 and equilibrated with 8M urea buffer pH7.5, and then the protein was loaded onto the column and washed with 50ml of 8M urea buffer pH7.5, 180ml of 25mM imidazole in urea buffer and 30ml of 40mM imidazole in urea buffer. The protein was finally eluted with 300mM imidazole in urea buffer.

The purity of the eluted protein was assessed by SDS-PAGE gel. The eluted fractions contained the protein as a single band, corresponding to the appropriate molecular weight, after staining with coomassie stain (Figure 4.1).

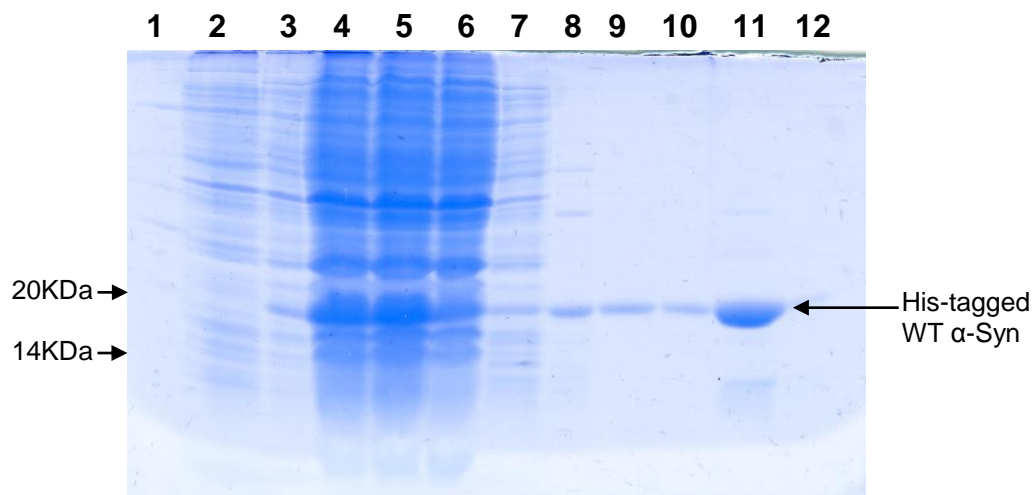


Fig. 4.1 Purified His-tagged WT α -Syn separated on a 14 % SDS-PAGE gel and coomassie stained. Lanes 1-12 represents: (1) protein marker (M), (2) un-induced sample (UI), (3) induced sample (I), (4) lysate (L), (5) filtrate (F), (6) flow-through (FT), (7) urea wash (Urea), (8) 25mM imidazole wash, (9) 40mM imidazole wash, (10-11-12) 300mM imidazole. Noting that majority of α -Syn is eluted in the second elution fraction (Lane 11).

The resultant His-tagged α -Syn was 95% pure after purification with almost no impurities. However, SDS gels occasionally revealed small bands alongside α -Syn band. Western blotting analysis was performed using α -Syn specific antibodies (Mouse anti- α -Syn, monoclonal antibody, Invitrogen) to detect the nature of these bands and whether they represent aggregates of α -Syn (Figure 4.2).



Fig. 4.2 Western blot analysis of purified his-tagged α -Syn after purification.

Western blotting showed only one band comprising his-tagged α -Syn. Other His-tagged α -Syn mutants, which lack the potential metal binding sites (H50A α -Syn and Δ 1-9 α -Syn), were also inserted into pTrc plasmid and purified similarly to His-tagged WT α -Syn (Figure 4.3). Protein identity was also performed routinely in the laboratory by western blotting. Protein concentration was determined by Bradford assay using a series of BSA standards, and measuring absorbance at 595nm. Prior to aggregation assays, the protein was dialysed in chelex-treated MilliQ water pH7, to remove any metal traces, at 4°C overnight.

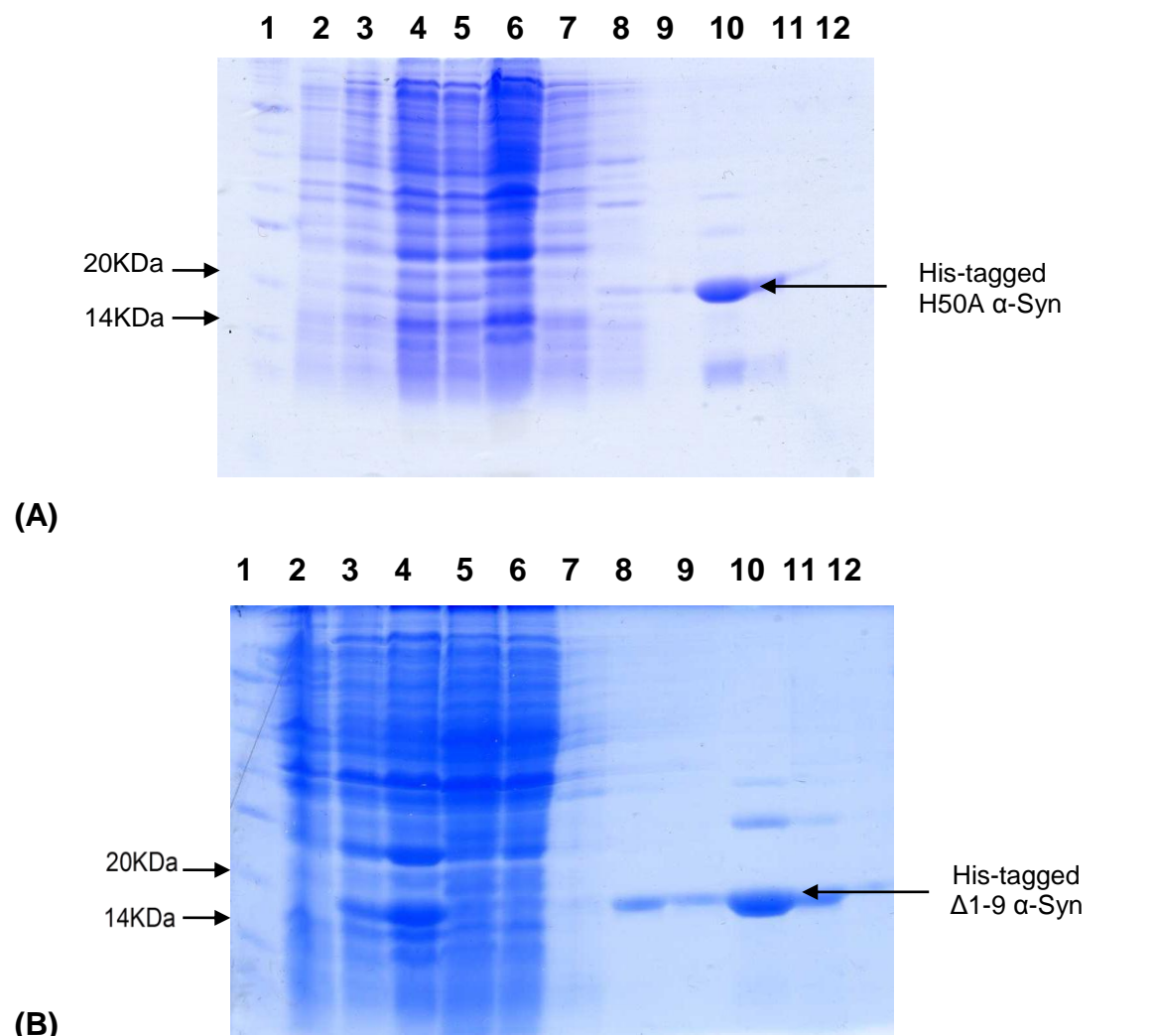


Fig. 4.3 Purified His-tagged α -Syn metal binding mutants separated on a 14 % SDS-PAGE gel and coomassie stained (A) His-tagged H50A α -Syn (B) His-tagged Δ 1-9 α -Syn. Lanes 1-12 represents: (1) protein marker (M), (2) un-induced sample (UI), (3) induced sample (I), (4) lysate (L), (5) filtrate (F), (6) flow-through (FT), (7) urea wash (Urea), (8) 25mM imidazole wash, (9) 40mM imidazole wash, (10-11-12) 300mM imidazole. Noting that majority of α -Syn is eluted in the first elution fraction (Lane 10). Proteins were dialysed against chelex-treated MilliQ water pH7 before using it in assays.

4.3 Aggregation Assay Optimisation

Primary aggregation experiments were conducted to assess the best conditions to be used for further experiments with metals. Optimisation experiments were all conducted using His-tagged α -Syn. WT α -Syn was prepared using the pET15b vector while both mutants H50A α -Syn and Δ 1-9 α -Syn were prepared using the pTrc vector.

4.3.1 Buffer and pH

Buffers were chosen based on those used in previous published studies (Uversky, Li et al. 2001b; Uversky, Li et al. 2002b; Rasia, Bertoncini et al. 2005; Bharathi and Rao 2007). There was a great variability in conditions as reported. However, two different buffers, Sodium Phosphate (NaPO_4) buffer and Tris-HCl buffer, were chosen. Both buffers were tested at pH6.5 and pH7.4. 10 μ M purified recombinant α -Syn was used with 10 μ M ThT in either buffer and then incubated with shaking at 37°C, 600rpm to induce aggregation. Fluorescence measurements were performed at different time intervals using a Fluoroskan Ascent Software version2.4. α -Syn aggregation occurred faster in the lower pH value and with enhanced level of ThT fluorescence. This is exhibited by higher fluorescence values reached by the blue line representative of the low pH value, as well as the less time taken for the fluorescence values to increase. ThT fluorescence values were normalized by subtracting the background fluorescence (buffer with ThT) and then as percentage against the highest value of each condition.

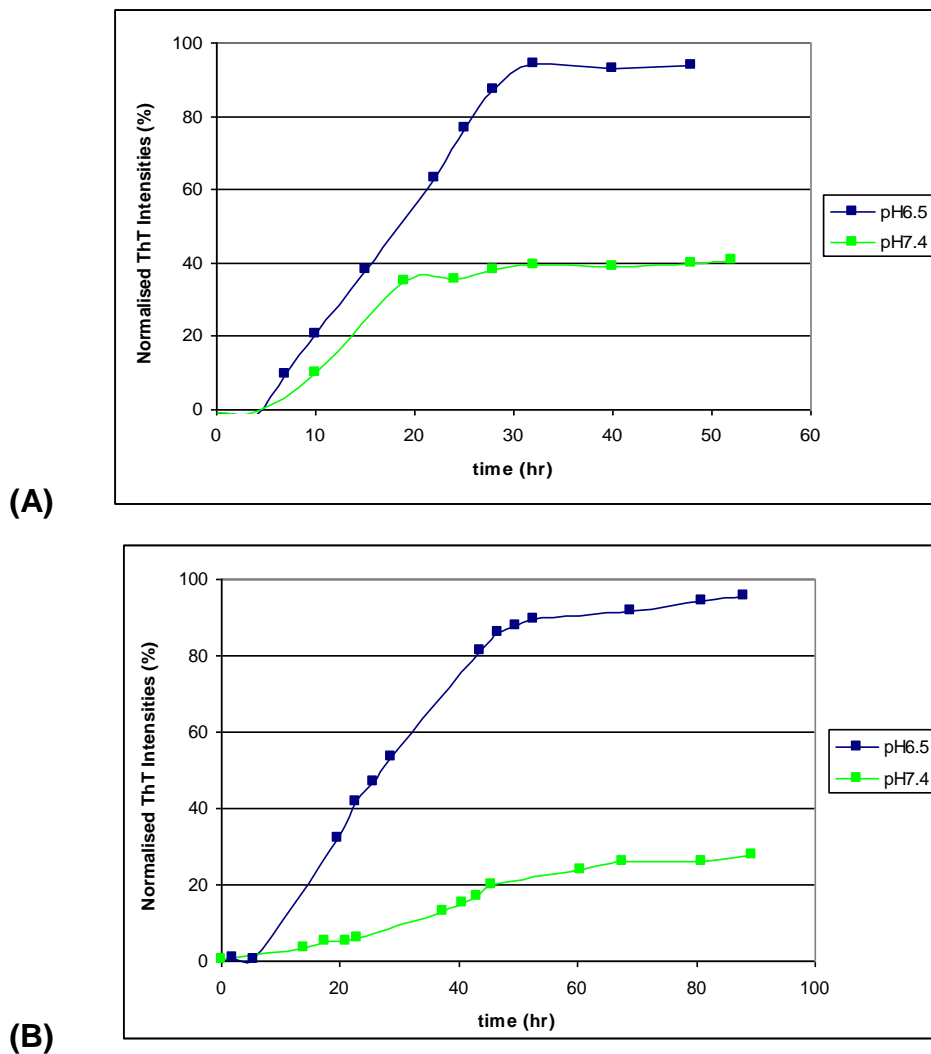


Fig. 4.4 Effect of buffer and pH on α -Syn aggregation kinetics. (A) 10 μ M α -Syn with 10 μ M ThT in sodium phosphate (NaPO_4) buffer at pH6.5 and pH7.4. (B) 10 μ M α -Syn with 10 μ M ThT in Tris buffer at pH6.5 and pH7.4. Measurements were performed with excitation at 444nm and emission at 485nm. Experiment solutions were incubated at 37°C with agitation at 600rpm. Values represent average of replicates in one experiment.

Figure 4.4 represents curves from an individual experiment. Time point values represent average of replicate within one experiment (wells replicate of each condition). Choosing buffer and pH value was important as α -Syn has a pI value of 4.7, therefore the lower the pH the faster it will aggregate. Also, different pH

values were reported to result in different aggregation kinetics and morphologies (Hoyer, Antony et al. 2002) where low pH values led to amorphous aggregates, rather than fibrils, to which ThT dye does not bind. Since faster aggregation was accompanied by lower pH value; therefore, pH7.4 was more favourable in addition to being more physiologically relevant. Comparing both buffers, it seemed that the aggregation pattern seen in Tris buffer pH7.4 represent a desirable kinetics to the type of experiments to be performed later in this work, as the rate was low to moderate and suitable to observe any occurring changes when adding metals. In addition, pH7.4 is more physiologically relevant than pH6.5.

4.3.2 Protein Concentration

This set of experiments was performed to confirm that α -Syn aggregation is a concentration dependent process (Uversky, Li et al. 2001b) and to enable the determination of an appropriate α -Syn concentration to be used in the following aggregation experiments. A range of α -Syn concentrations were used in ThT assays in both 10mM Tris buffer pH7.4 and 10mM Sodium Phosphate buffer pH7.4. As the aim was to look for protein concentration that enables the aggregation to occur at relatively slow yet detectable rate, α -Syn concentrations used started at 1 μ M up to 10 μ M (Figure 4.5). Protein concentration was assessed using Bradford assay. α -Syn at 1 μ M had minimal aggregation as shown by hardly detectable ThT fluorescence with very low ThT fluorescence intensity values. α -Syn aggregation showed a long lag phase occurring over an observable amount of time with 5 μ M and 10 μ M concentration but with better fluorescence, pre-normalized values, with the higher concentration. Therefore, 10 μ M was chosen to be the protein concentration used in aggregation assays.

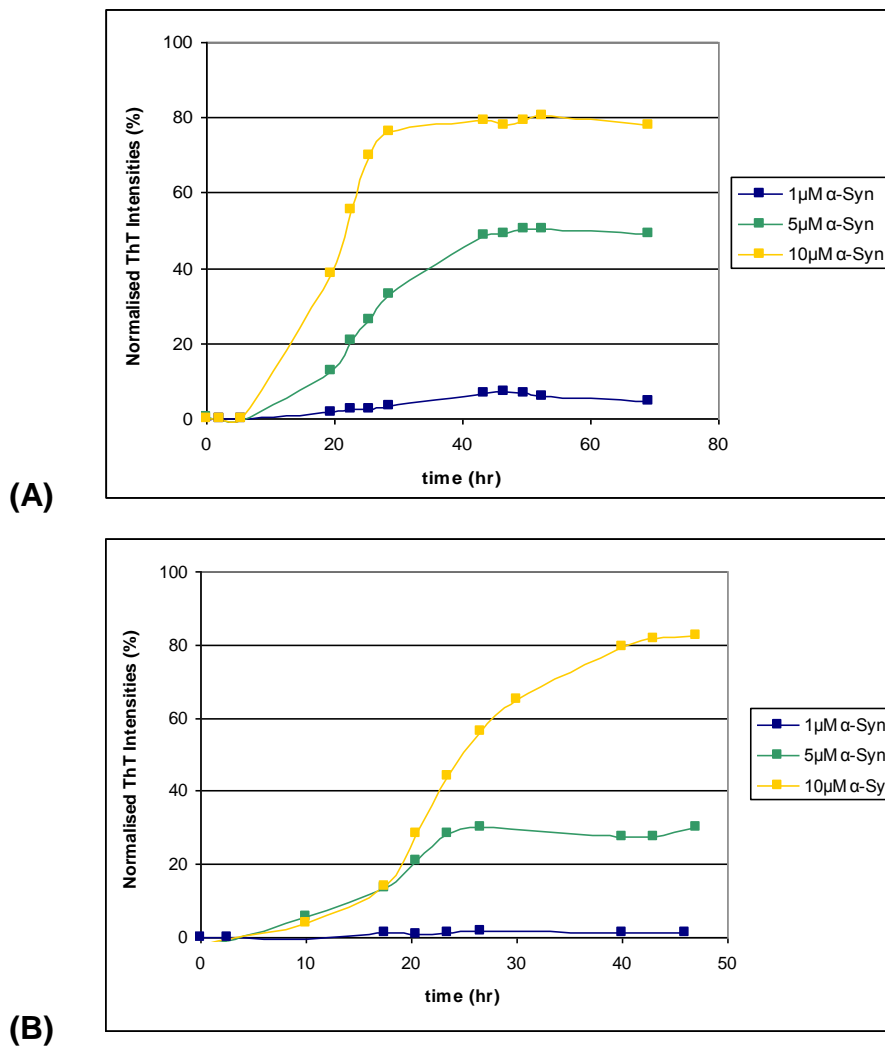


Fig. 4.5 The effect of protein concentration on α -Syn aggregation kinetics. Purified α -Syn was diluted with either 10mM NaPO₄ pH7.4 (A) or with 10mM Tris pH7.4 (B) to obtain a range of final protein concentrations of 1 μ M and 5 μ M in a 100 μ l final volume and liquated into 96-well plate. Kinetics of aggregation was monitored by ThT Fluorescence. Measurements were performed with excitation at 444nm and emission at 485nm. Experimental plate was incubated at 37°C, shaking at 600rpm. Curves shown in this figure represent one experiment. Values represent average of replicates in one experiment.

Also, the figure above shows that α -Syn aggregation occurred faster in Sodium Phosphate buffer pH7.4 comparing to Tris buffer at 5 μ M and 10 μ M final

concentration, which emphasizes the result shown in figure 4.4, that Tris buffer at pH7.4 provides favourable conditions for later experimental work.

4.4 Effect of Metals on His-tagged α -Syn Aggregation

Aggregation experiments assessing the effect of metals on His-tagged proteins were conducted using WT α -Syn, H50A α -Syn and its N-terminal mutant (Δ 1-9 His-tagged α -Syn). Each protein was used at 10 μ M final concentration in 10mM Tris pH7.4, incubating at 37°C, and shaking constantly at 600rpm with or without metals (Cu^{2+} , Fe^{3+} , and Al^{3+}) at 100 μ M final concentration. After purification and dialysis, protein concentration used in these experiments was measured using Bradford assay. Samples were run in quadruplicates and ThT fluorescence intensities were recorded at different time points. Quadruplicates were averaged and plotted as a function of time. Similar to previous reports (Uversky, Li et al. 2001a), α -Syn aggregation kinetics were found to be sigmoidal (Figure 4.4 and 4.5), defined by an initial lag phase where no changes in ThT intensities are observed, a subsequent growth phase in which ThT intensities increase and a final equilibrium phase in which ThT fluorescence reaches a plateau indicating the end of β -sheet rich fibril formation.

4.4.1 Effect of metals on His-tagged WT α -Syn

Aggregation assays were performed using 10 μ M final protein concentration in 10mM Tris buffer pH7.4 mixed with 10 μ M ThT, incubated at 37°C and shaking constantly at 600rpm with and without metals at 100 μ M final concentration. Copper was the most effective metal ion in terms of inducing the aggregation of His-tagged WT α -Syn. This is illustrated in the following figure by having the highest ThT fluorescence values and the least time taken to initiate aggregation comparing to iron and aluminium. Less effect was observed with iron and

aluminium, represented by orange and green lines respectively, as demonstrated by lower ThT Fluorescence intensities measured during the course of experiment and longer lag phase (Figure 4.6).

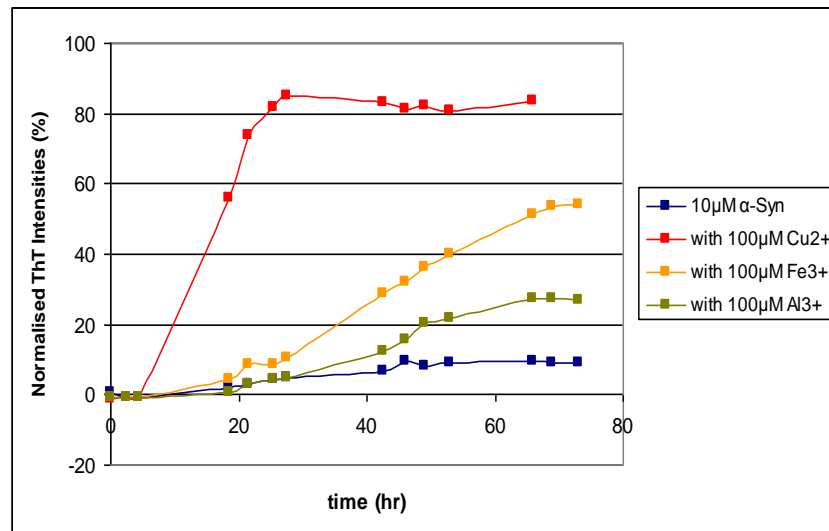


Fig. 4.6 Effect of metals on His-tagged WT α -Syn aggregation kinetics. 10 μ M WT α -Syn with or without 100 μ M metals was incubated at 37°C, shaking constantly at 600rpm. ThT measurements were recorded at designated time points. Values represent average of replicates in one experiment.

Fluorescence intensity of 10 μ M α -Syn in the absence of metals (Figure 4.5) was ~80%, while less 20% in Figure 4.6 where metals were present. This is due to the use of the highest fluorescence intensity value obtained in each experiment individually to normalise the rest of the ThT fluorescence values as a percentage of that high value. This value differed between both experiments and was much higher when copper was present with α -Syn.

Acceleration of α -Syn aggregation induced by metals exceeded that arising from α -Syn with no metals shown by the blue dots and the blue line in Figure 4.6. Copper came first and caused the highest level of acceleration. Iron and

aluminium came second and third respectively and also resulted in higher ThT intensities and shorter lag time compared to the case where no metals were present with α -Syn.

Both his-tagged α -Syn mutants, which lack the potential metal binding sites (H50A α -Syn and Δ 1-9 α -Syn), were inserted into a different his-tag vector (pTrc plasmid vector) to WT α -Syn and purified in the same way. Protein concentration was determined by Bradford assay using a series of BSA standards, and measuring absorbance at 595nm. Prior to aggregation assays, the protein was dialysed in chelex-treated MilliQ water pH7, to remove any metal traces, at 4°C overnight. In order to validate the use of the mutant proteins in a different plasmid vector, an aggregation experiment was run to compare WT α -Syn purified from both plasmid vectors (pET15b and pTrc) and another comparing WT α -Syn with its his-tagged mutants (Figure 4.7).

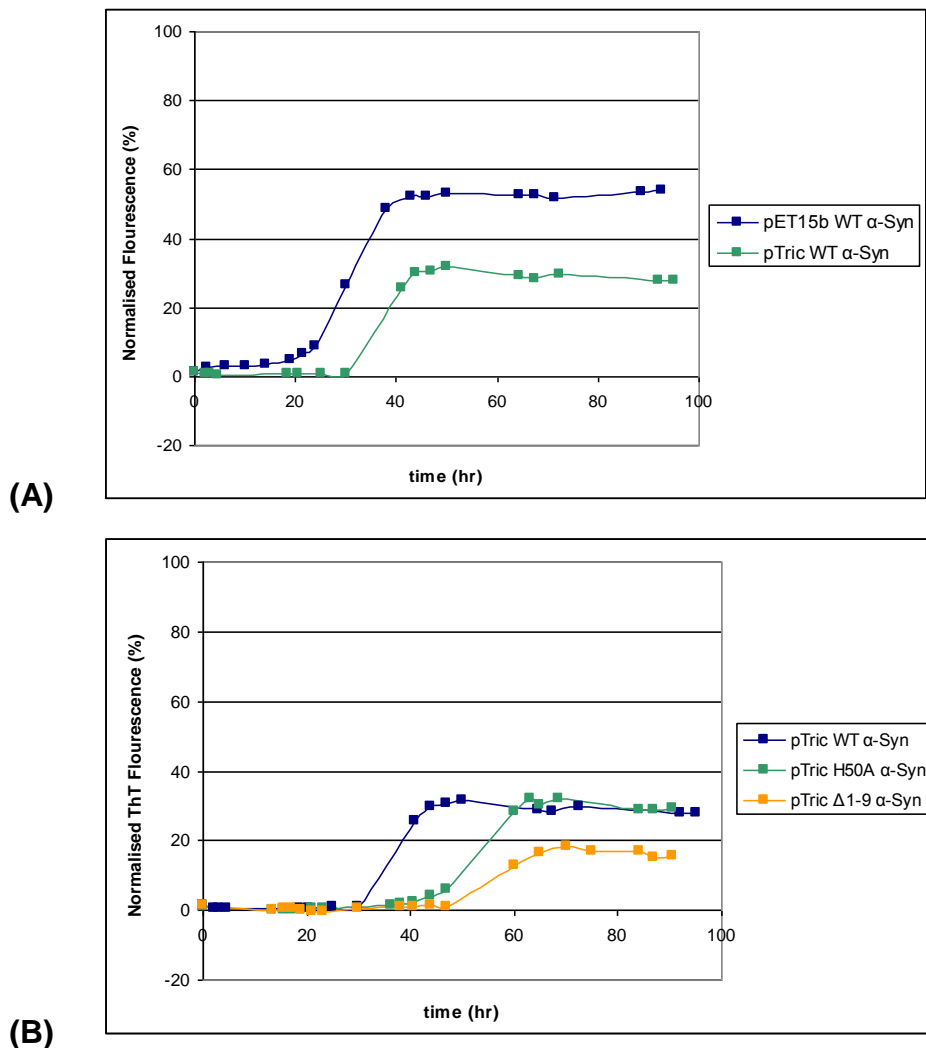


Fig. 4.7 (A) His-tagged α -Syn aggregation kinetics in relation to the plasmid vector inserted in. His-tagged WT α -Syn inserted into either pET15b or pTric vector. (B) Comparison of the aggregation pattern of WT α -Syn and metal binding mutants all in pTric vector. Values represent average of replicates in one experiment.

Results shown in Figure 4.7 above demonstrate that α -Syn in pTrc plasmid vector has slightly slower aggregation to α -Syn in pET15b vector as shown by lower fluorescence intensities and longer lag time (A). Yet it can be seen that α -Syn in both plasmids reached its maximum fluorescence levels around the same time. In addition, the second graph (B) shows that the eventual extent of aggregation of WT α -Syn is fairly similar to metal binding mutants (H50A and Δ 1-

9 α -Syn) as shown by maximal ThT fluorescence. Although the figure indicates that WT α -Syn reaches the maximum fluorescence relatively faster than the mutant proteins. However, the longer lag phase of mutants shown in the above figure represents an anomalous single experiment which was not present in all the repeats and such a difference when present does not influence any potential effect metals can have when added to each of the tested proteins.

4.4.2 Effect of metals on His-tagged Mutants of α -Syn

Aggregation assays of α -Syn mutants were performed similar to His-tagged WT α -Syn, using 10 μ M final concentration of His-tagged mutant protein in 10mM Tris buffer pH7.4 mixed with 10 μ M ThT, incubated at 37°C and shaking constantly at 600rpm with and without adding metals at 100 μ M final concentration. As copper was the most effective metal in inducing WT α -Syn aggregation, it was solely tested with His-tagged H50A α -Syn and Δ 1-9 α -Syn. Samples were assayed in quadruplicates. ThT measurements were recorded at designated time point, averaged and plotted as function of time (Figure 4.8).

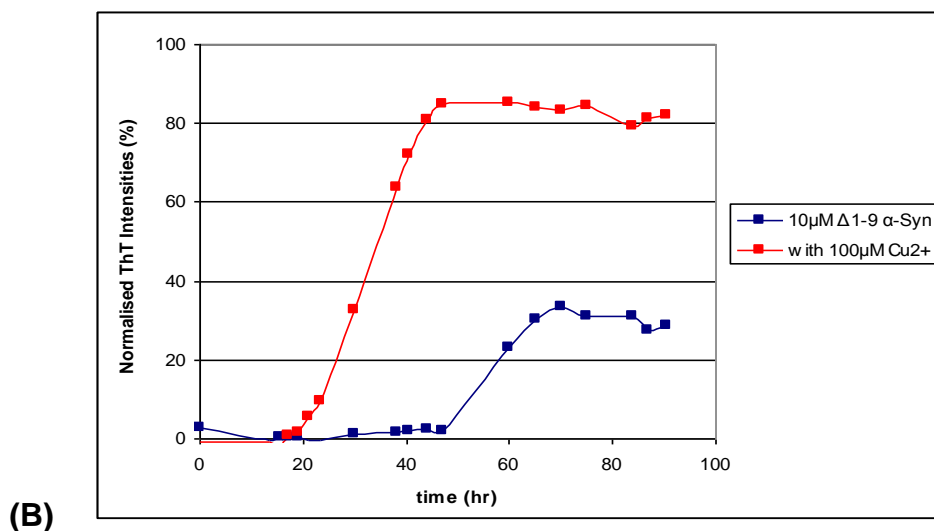
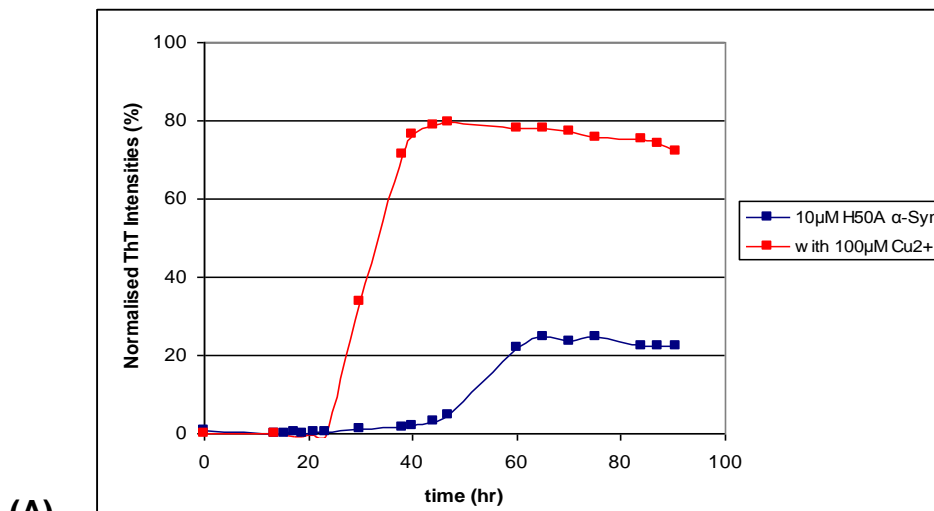


Fig. 4.8 Effect of copper on the aggregation kinetics of α-Syn His-tagged metal binding mutants (A) His-tagged H50A α-Syn, (B) His-tagged Δ1-9 α-Syn. 10µM protein was mixed with 10µM ThT and incubated with and without 100µM Cu²⁺ at 37°C, shaking constantly at 600rpm. ThT measurements were recorded at designated time points. Values are average of replicates in one experiment.

Copper resulted in considerable and comparable acceleration of the aggregation kinetics of both α-Syn metal binding mutants compared to the case where no copper is present. This is shown from the lag time where ThT fluorescence of both proteins reached maximum values at about 40hr. Both mutants are his-tagged protein and lack a number of amino acids at potentially copper binding

sites (H50A or amino acids 1-9). Lacking either of the potential copper binding sites resulted in no major difference on the observed aggregation when copper was added to each protein.

4.5 Effect of Metals on Untagged α -Syn Aggregation

After the purification method of untagged proteins had been designed, untagged α -Syn was purified according to the protocol described in the materials and methods chapter. Untagged proteins purified included WT α -Syn and mutants lacking the potential metal binding sites such as H50A α -Syn, Δ 1-9 α -Syn, and Δ 1-9 H50A α -Syn (double mutant). Ahead of conducting aggregation experiments of untagged α -Syn with metals, the protein was used in a primary aggregation experiment against His-tagged α -Syn to compare their aggregation patterns. Samples were run in quadruplicates and incubated in 96-well plates shaking constantly at 37°C, 600rpm. ThT fluorescence intensities were measured at different time points. Quadruplicates were averaged, plotted as a function of time. The aggregation of His-tagged α -Syn occurs faster than untagged α -Syn as indicated by the higher fluorescence intensities and shorter lag time shown in Figure 4.9.

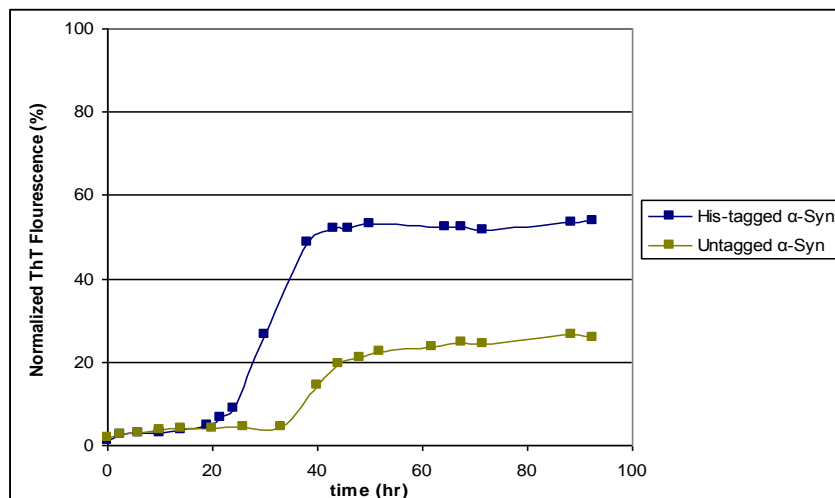


Fig. 4.9 Comparison of the aggregation patterns of His-tagged and untagged α -Syn. 30 μ M α -Syn was incubated in 10mM Tris pH7.4 at 37°C, shaking constantly at 600rpm. Samples were run in quadruplicates. ThT fluorescence intensities were recorded at different time points and plotted as a function of time. Values are average of replicate wells in one experiment.

The result in Figure 4.9 comes from an individual experiment where values of ThT intensities at each time point represent the average of wells replicate. The pattern of faster aggregation of His-tagged α -Syn compared to untagged α -Syn was also observed when repeating the experiment. In addition to the higher ThT intensities, the slope of the curve is steeper with His-tagged α -Syn. Moreover, calculating the rate of both forms of the protein showed higher rate of 0.884 (h^{-1}) for His-tagged α -Syn, comparing to 0.357 (h^{-1}) of untagged α -Syn which is consistent with what the Figure 4.9 shows of faster aggregation of His-tagged α -Syn.

4.5.1 Effect of Metals on Untagged WT α -Syn

Untagged WT α -Syn was used at a final concentration of 10 μ M, similar to experiments performed with His-tagged proteins, with and without 100 μ M final

concentration of each metal. The three metals tested (Cu^{2+} , Fe^{3+} and Al^{3+}) resulted in acceleration of the aggregation of untagged WT α -Syn, with ferric iron and copper being slightly more effective comparing to aluminium (Figure 4.10). This is due to observation that α -Syn aggregation and increased ThT fluorescence started to occur faster when copper and iron were present and to less extent with aluminium.

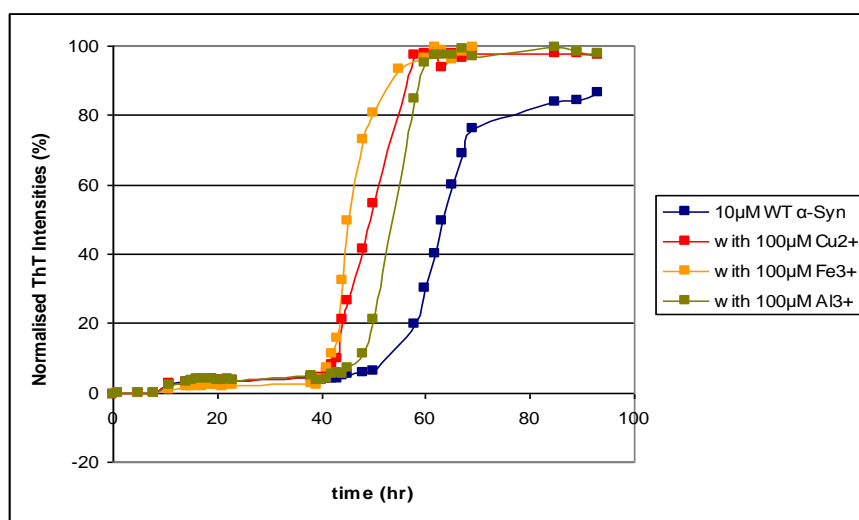


Fig. 4.10 Effect of metals on the aggregation kinetics of WT α -Syn. 10μM WT α -Syn was incubated in 10mM Tris pH7.4 with or without 100μM metals at 37°C, shaking constantly at 600rpm. Samples were run in quadruplicates and aliquoted in 96-well plate at a total volume of 100μl in each well. ThT fluorescence intensities were recorded at different time points and plotted as a function of time. Values represent average of replicates in one experiment.

Higher ThT fluorescence intensities and faster occurrence of aggregation were obtained in the presence of metals as ThT fluorescence started to increase at around 40hrs. This is further analysed by calculating the apparent rate constant (k_{app}) of α -Syn when incubated alone and with metals (Figure 4.11).

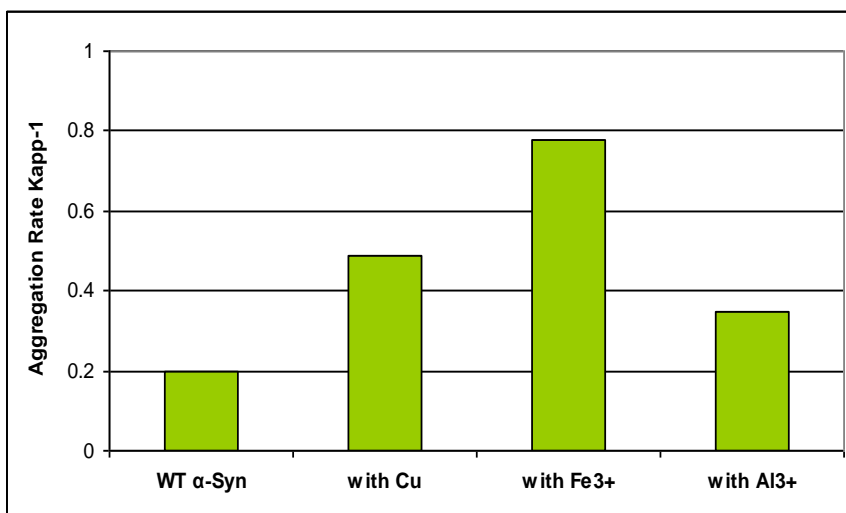


Fig. 4.11 Plot of the aggregation rate (k_{app}) (h^{-1}) obtained for untagged WT α -Syn in the presence of 100 μ M metals. Data presented in this figure represent one experiment.

Figure 4.11 shows that ferric iron and copper caused more than 2-fold increase in the aggregation rate of untagged WT α -Syn which emphasizes the results observed with ThT assays. Aggregation rate (k_{app}) was calculated using SigmaPlot from data obtained from one experiment (Section 2.2.11 Materials and Methods). This trend was confirmed with subsequent experiments. Copper, in four other experiments, showed similar acceleration of α -Syn aggregation. The rate was calculated in each experiment and resulted in an average of 1.6 fold increases with a standard deviation of 0.123 caused by copper.

However, it was difficult to get more successful repeats of the experiment. There is no single reason for the failure in achieving more working repeats. It may result from various causes such as protein preparation and process with its accompanied different stages that could affect protein produce in terms of action and folding. The following figure (Figure 4.12) shows a representative experiment

where no aggregation was observed with WT α -Syn in the absence and the presence of metals. Over time, no increase in ThT fluorescence occurred.

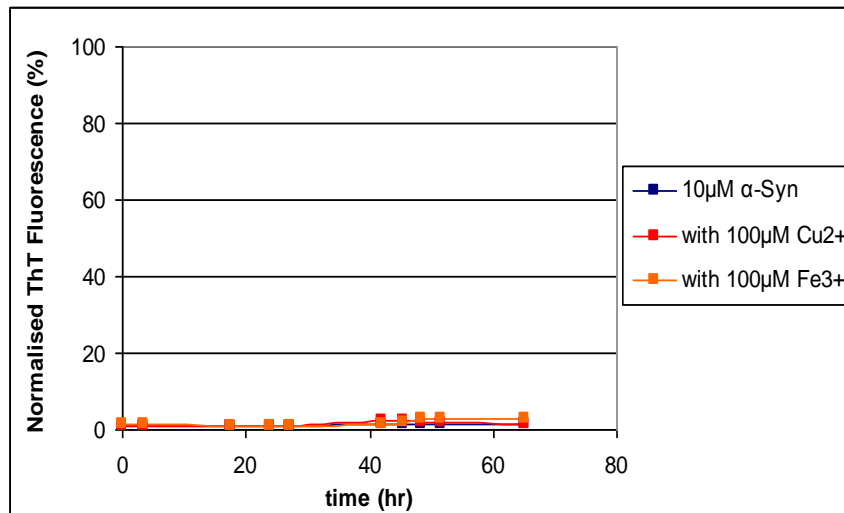


Fig. 4.12 Untagged WT α -Syn aggregation in the presence of metals where no effect was observed. 10µM WT α -Syn with or without 100µM metals was incubated at 37°C, shaking constantly at 600rpm. ThT measurements were recorded at designated time points. Values represent average of replicates in one experiment.

There was no producibility of results when assessing WT α -Syn aggregation despite more than 10 attempts undertaken to correct this condition and overcome problems associated with this assay. All except one aggregation experiment showed no increased ThT fluorescence. Although the number of negative experiments was high, the positive results shown in the single experiment would be valid since similar results were published in the literature (Uversky, Li et al. 2001b). Hence, data were shown as only one representative experiment for each protein.

4.5.2 Effect of Metals on Untagged α -Syn Mutants

To further assess the role of metals in inducing the aggregation of α -Syn, mutants of the protein lacking the potential metal binding sites were created and purified similarly to WT α -Syn and used in aggregation assays. These mutants included the H50A α -Syn, the N-terminal mutant (Δ 1-9 α -Syn), and a second N-terminal mutant (Δ 1-9 H50A α -Syn). Both N-terminal mutant proteins were used at 10 μ M final concentration in 10mM Tris pH7.4, and mixed with 10 μ M ThT. 100 μ M of each metal was then added and samples were incubated at 37°C, shaking constantly at 600rpm (Figure 4.13). Both ferric iron and copper accelerated the aggregation of both α -Syn mutants at 100 μ M final metal concentration and aluminium to a lesser degree as illustrated by the shorter time taken to reach the maximum fluorescence. Iron being the most potent accelerator. This is shown in the following figure by the orange and red lines representing the aggregation of α -Syn mutants in the presence of iron and copper, respectively.

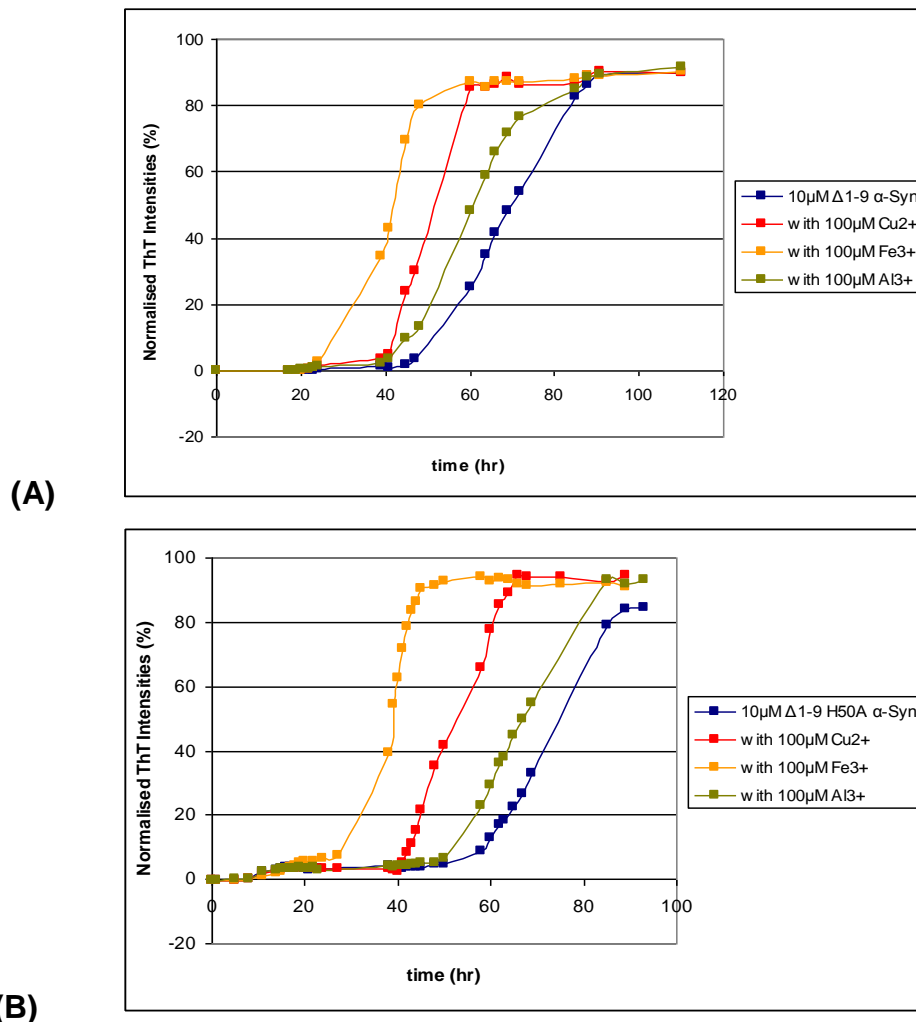


Fig. 4.13 Effect of metals on the aggregation kinetics of the untagged metal binding mutants of α-Syn (A) Δ1-9 α-Syn and (B) Δ1-9 H50A α-Syn. 10μM protein was incubated in 10mM Tris pH7.4 with or without 100μM metal at 37°C, shaking constantly at 600rpm. ThT fluorescence was recorded at different time points and plotted as function of time. Values represent average of replicates in one experiment.

The result observed with the mutant proteins resembles that of WT α-Syn with ferric iron being the most effective metal in enhancing the aggregation of the protein. Similarly to WT α-Syn, the result was confirmed by calculating the aggregation rate (k_{app}) using SigmaPlot (Figure 4.14).

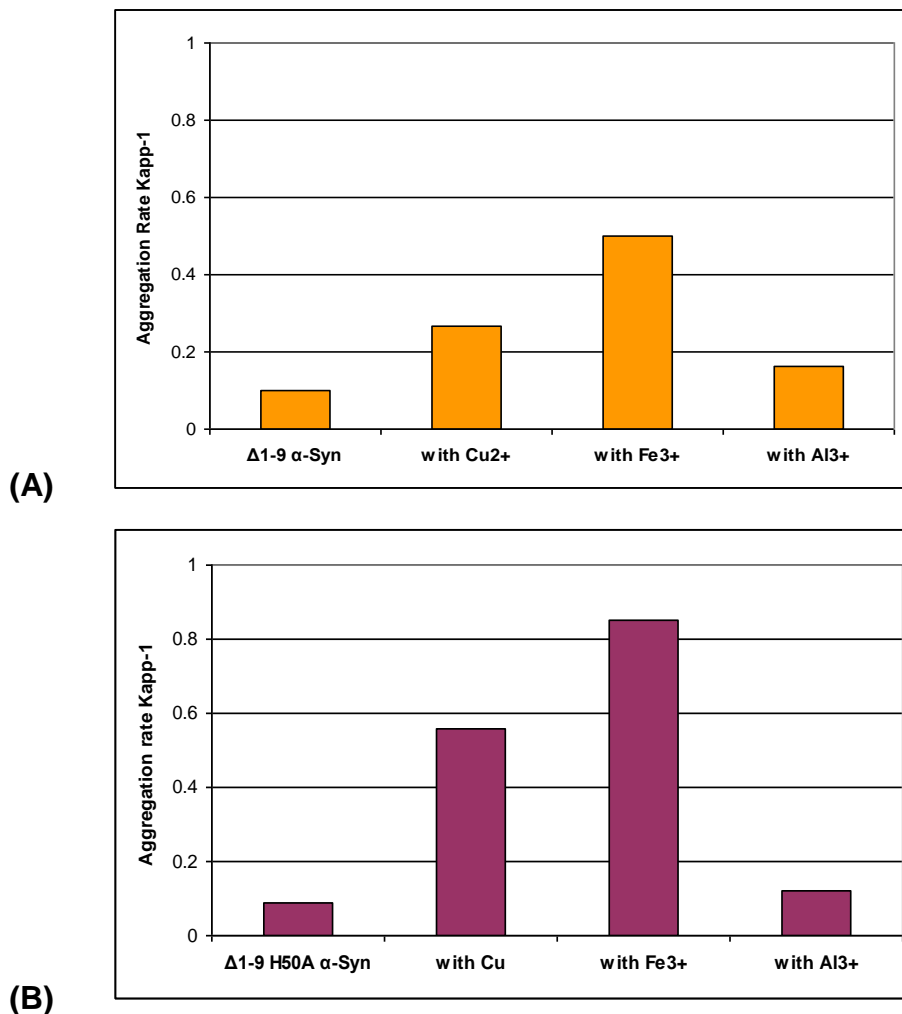


Fig. 4.14 Plot of the aggregation rate (k_{app}) obtained for untagged metals binding mutants of α -Syn (A) Δ 1-9 α -Syn and (B) Δ 1-9 H50A α -Syn, in the presence of 100 μ M metals. Data presented in this figure represent one experiment.

Some level of variability was present while conducting the aggregation experiments. While the trends were reproducible, the rates of aggregation varied. Different untagged protein batches were tested for their ability to aggregate. At this stage, inaccuracies were discovered in the Bradford assay for protein quantification when applied to α -Syn. Bradford assay was found to underestimate α -Syn concentration by approximately 10 fold when comparing resultant concentration to that of A_{275} . Therefore, aggregation experiments of H50A α -Syn were carried out at 100 μ M final protein concentration which resembles the

content of 10 μ M protein estimated at previous work stages via Bradford assay. The concentrations of metals were kept at 100 μ M to keep it relevant to previous experiments. This was accompanied by the change into the use of MOPS buffer instead of Tris buffer mainly since Tris buffer can interact with copper (Smith and Martell 1987). Other experimental conditions were similar such as dialyse the protein in chelex-treated MilliQ water pH7 prior to utilising it in aggregation assay. Protein samples with or without metals were aliquoted into 96-well plates in a final volume of 100 μ l. Samples were run in triplicates and incubated at 37°C, shaking constantly at 600rpm. Results of H50A α -Syn ThT assay are shown in the following (Figure 4.15). Copper had the largest effect on accelerating the aggregation of H50A α -Syn. Fluorescence intensities obtained with copper were much higher compared to iron, in addition to where no metal was present. Also less time was taken to get increased ThT fluorescence.

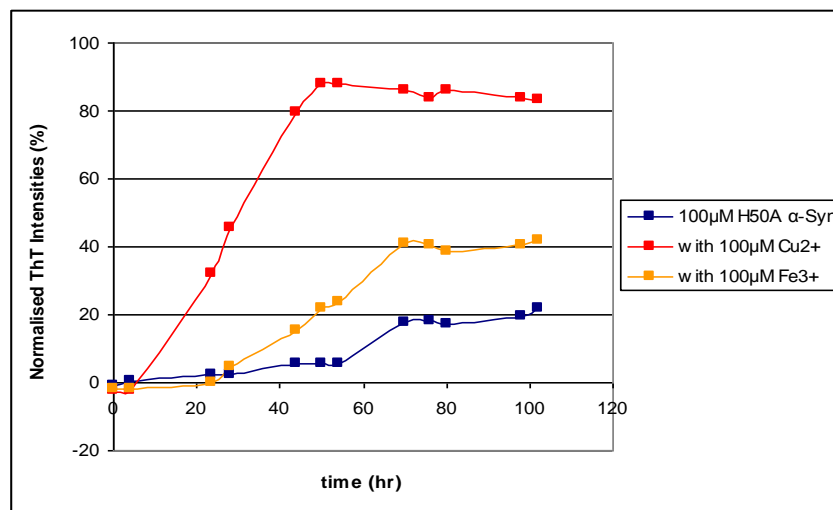


Fig. 4.15 Effect of metals on the aggregation kinetics of H50A α -Syn. 100 μ M protein was incubated in 10mM MOPS pH7.4 with or without 100 μ M metal at 37°C, shaking constantly at 600rpm. ThT fluorescence intensities were recorded at different time points and plotted as function of time. Values represent average of replicates in one experiment.

Both metals (Cu^{2+} and Fe^{3+}) caused enhancement in H50A α -Syn aggregation, but at much higher level with Cu^{2+} comparing to Fe^{3+} . This was shown in terms of maximum fluorescence intensities reached and time taken to achieve it.

4.6 Alpha Synuclein Aggregation in Cell Culture

Cell culture and animal models have been used to study the aggregation of α -Syn and its consequences on cell viability including the identification of the possible toxic species. α -Syn in an oligomeric or aggregated form was shown to be toxic (Danzer, Haasen et al. 2007; Wright, Wang et al. 2009). Here in this work, neuroblastoma cells (SHSY-5Y cells) were utilised to assess the aggregation and oligomer formation of α -Syn in response to metals treatment. Stable cell lines of SHSY-5Y over-expressing WT α -Syn, metal binding mutants ($\Delta 1-9$ α -Syn, H50A α -Syn, and $\Delta 119-126$ α -Syn) and disease associated mutants (A30P, A53T and E46K) were generated. SHSY-5Y cells were individually transfected with each of those proteins to over-express them and produce stable cell lines, in a way to verify that aggregates increase upon exposure to metals (Figure 4.16).

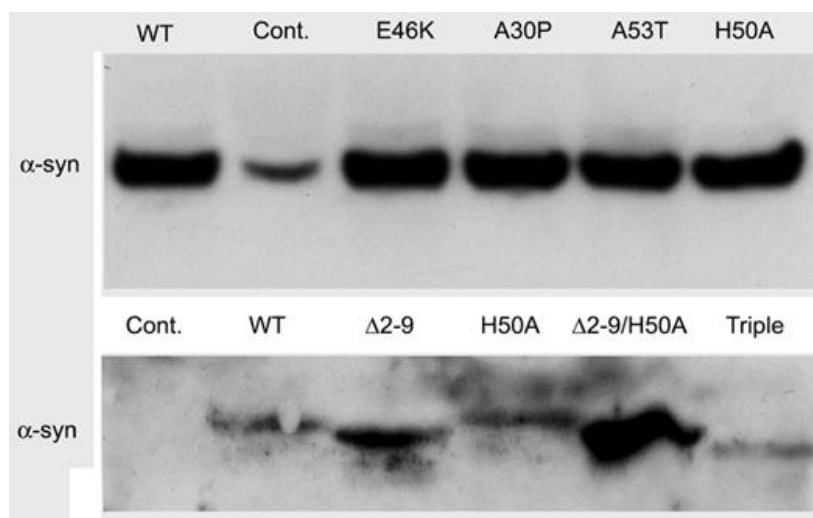


Fig. 4.16 Analysis of stably transfected SHSY-5Y cells for the level of protein expression. SHSY-5Y cells were transfected with pcDNA3.1 or the plasmid with the ORF of WT α -Syn or its mutants. Protein extracts were prepared from cells and electrophoresed on 12% acrylamide PAGE gels followed by western blotting. Protein bands corresponding to α -Syn were shown using immunodetection via a specific antibody for α -Syn. Cont. refers to pcDNA3.1 plasmid and Triple refers to Δ 2-9 H50A Δ 119-126 α -Syn.

Figure 4.16 shows increased level of expression of the proteins compared to pcDNA3.1 transfected cells (control) using two anti- α -Syn antibodies. The first line represent cells immunodetected with an antibody directed against the C-terminus of α -Syn, while the second line represents cells immunodetected with an antibody directed against amino acids 11-26. Control cell lines are cells transfected with pcDNA3.1 with no protein expressing insert in it. α -Syn corresponding band was found in all cell lines including the control which represent endogenous α -Syn in cells. Cells were grown in 6-well plates in either normal media or chelex-treated media prior to metal treatment (10 μ M, 100 μ M or 300 μ M). After 48hr cells were harvested, lysed and equal amounts of total protein were then loaded onto a reducing gradient gel as estimated by Bradford assay. Protein bands corresponding to monomeric or oligomeric α -Syn were revealed using western blotting immunodetection using α -Syn specific antibodies.

The same procedure was applied for the different proteins tested. Equal protein loading was confirmed using an antibody for tubulin (Sigma).

Oligomers (~150kD) were seen in all cell extracts regardless of the treatment, which could also represent endogenous level of α -Syn oligomers. In the case of WT α -Syn, there were significantly increased levels of oligomers in cells treated with copper but not iron (Figure 4.17). Intensities of aggregate bands were compared between different conditions using densitometry (Figure 4.18).

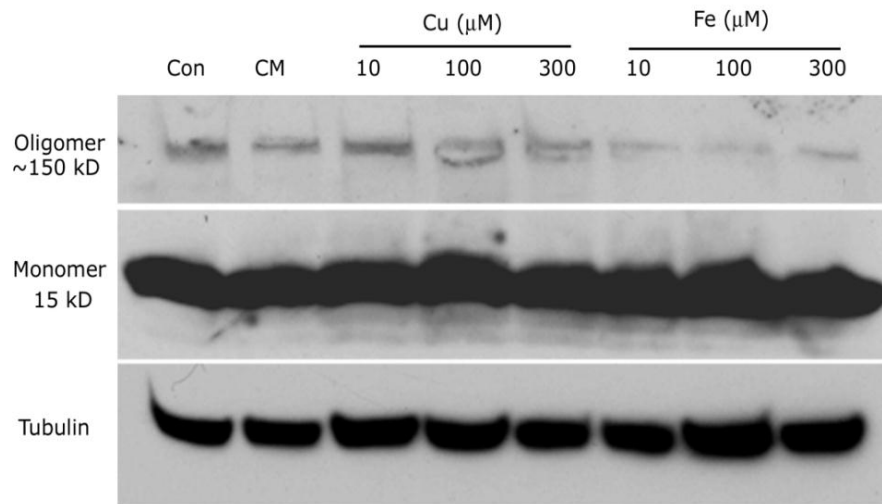


Fig. 4.17 SHSY-5Y cells stably transfected with WT α -Syn were grown in normal media(Con), chelex-treated media (CM) and with metals added. Representative experiment of protein separated in 5-20% gradient gel. Western analysis was performed using α -Syn or tubulin antibodies. Lanes on gradient gel shows the different conditions starting with: normal media (Con), chelex-treated media (CM), 10 μ M Cu²⁺, 100 μ M Cu²⁺, 300 μ M Cu²⁺, 10 μ M Fe³⁺, 100 μ M Fe³⁺, 300 μ M Fe³⁺.

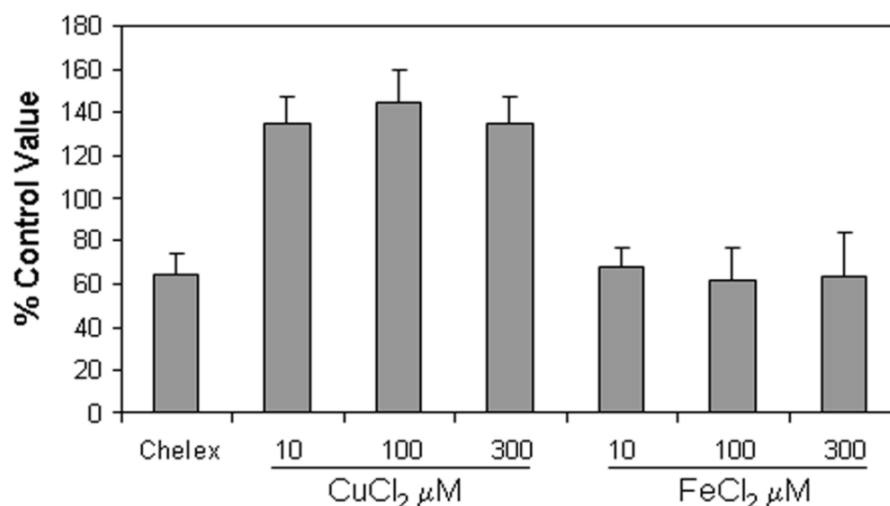


Fig. 4.18 Integrated density measurements of higher molecular weight α -Syn aggregates in SHSY-5Y cells stably transfected with WT α -Syn and grown in normal media, chelex-treated media and with metals added. Density measurements were related to the untreated control (normal media) as metals were added to cells grown in chelex-treated media. Data from three independent experiments were pooled and is represented as mean \pm standard error. Statistical analysis was performed using Student-t test. $P < 0.05$ chelex-treated versus 10 μ M Cu^{2+} , 100 μ M Cu^{2+} and 300 μ M Cu^{2+} .

Similar experiments were performed with SHSY-5Y cells expressing each of α -Syn mutants. Cells were grown in 6-well plates and treated with different concentrations of Cu^{2+} and Fe^{3+} , and harvested 48hrs later for analysis. A similar pattern was noted in the case of α -Syn disease-associated mutants (A30P, A53T and E46K); to that obtained with WT α -Syn (Figure 4.19). Each of the disease-associated mutants resulted in an increase in oligomeric aggregates particularly with 100 μ M Cu^{2+} treatment, but not greater than that seen with WT α -Syn. On the other hand, no such increase in the level of oligomers was observed with cells expressing α -Syn copper binding mutants in response to copper (Figure 4.19).

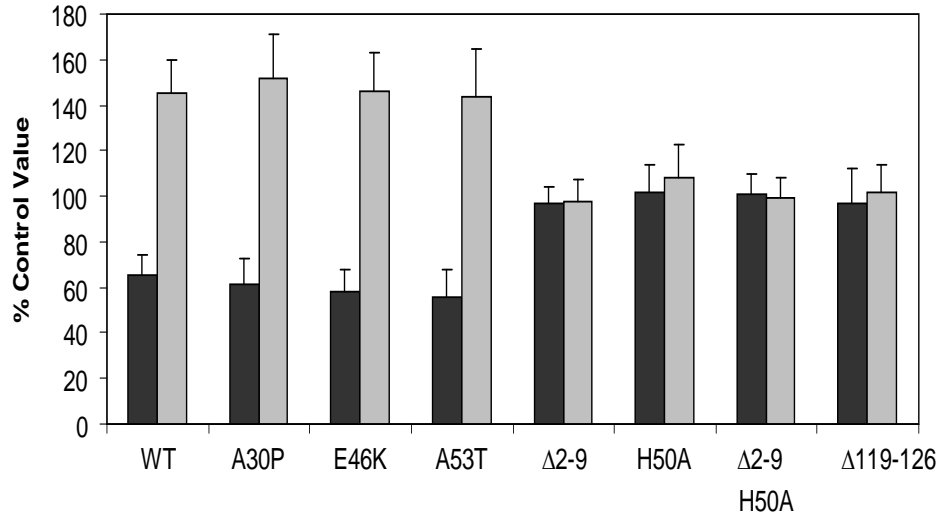


Fig. 4.19 The effect of 100µM Cu²⁺ on aggregates formation of α-Syn mutants. SHSY-5Y cells expressing each of the proteins were grown in 6-well plates and treated with 100µM Cu²⁺ for 48hrs. Western blot analysis was performed using specific α-Syn and α-tubulin antibodies. Higher molecular weight oligomeric species of α-Syn were quantified densitometrically and compared to the untreated control. Data from three independent experiments were pooled and presented as mean ± standard error. The presence of Cu²⁺ caused significant increase in the level of oligomeric aggregates of WT α-Syn and its disease-associated mutants using Student's t test (p<0.05). Black bars represent cells over-expressing protein in chelex-treated media. Grey bars represent cells over-expressing protein in chelex-treated media in the presence of 100µM Cu²⁺.

The results shown in both figures (Figure 4.17, Figure 4.18 and Figure 4.19) confirm that deletion of copper binding domains in α-Syn prevented copper from stimulating aggregation of the protein within cells.

4.7 Discussion

α-Syn aggregation has been recognized as a pathological feature of PD and other neurodegenerative diseases (Spillantini, Schmidt et al. 1997). Since it has

been discovered, it gained a lot of interest as to reveal causative factors and the mechanism by which it occurs. In this regard metals have attracted increased attention given that iron deposits have been discovered in Lewy bodies (Castellani, Siedlak et al. 2000) and elevated concentrations of copper have been reported in PD patients (Pall, Williams et al. 1987). Metals have been shown to enhance protein aggregation in other neurodegenerative diseases such as PrP in prion disease (Brown, Guantieri et al. 2004) and A β in Alzheimer's disease (Bush, Masters et al. 2003). Both proteins have also been identified as metal-binding proteins especially to Cu²⁺ (Hesse, Beher et al. 1994; Viles, Cohen et al. 1999; Thompson, Abdelraheim et al. 2005), which directed research to investigate the ability of α -Syn to bind metals. As such, copper binding to α -Syn was widely assessed, and α -Syn was identified as a copper binding protein (Rasia, Bertoncini et al. 2005). In addition to copper, α -Syn was found to bind iron and other divalent metal ions such as Fe²⁺ and Mn²⁺ (Binolfi, Rasia et al. 2006). Furthermore, this study has shown ferric iron (Fe³⁺) binding properties to α -Syn. Copper and iron, have not only been shown to bind to α -Syn, but also to accelerate its aggregation (Uversky, Li et al. 2001b), even though the mechanism is not yet known. A direct interaction between α -Syn and the metals has been proposed to be the underlying mechanism of the aggregation (Rasia, Bertoncini et al. 2005). Also, metal binding has been proposed to strongly influence the fibrillation process as it could lead to electrostatic masking of the negative charges at the C-terminus of α -Syn (Bharathi and Rao 2007), and could cause perturbation of the N-terminus long range interactions (Rasia, Bertoncini et al. 2005; Binolfi, Lamberto et al. 2008), since the main metal binding takes place at the N-terminus. Based on that, mutants of α -Syn lacking the potential metal binding sites at the N-terminus (residues 1-9) and His-50 individually and combined (Δ 1-9H 50A α -Syn) were created. α -Syn was assessed as either His-tagged or untagged forms. Thioflavin-T (ThT) assay was used to assess α -Syn aggregation and fibril formation as previously reported for the same purpose (Uversky, Li et al. 2001b; Uversky, Li et al. 2002b; Khan, Ashcroft et al. 2005; Rasia, Bertoncini et al. 2005; Binolfi, Rasia et al. 2006).

While the method of purification of untagged protein was still being developed in our laboratory, aggregation experiments (ThT assays) were performed to give a preliminary assessment using His-tagged protein. His-tagged α -Syn was produced via two different vectors, pET15b and pTrc vectors. Either vector generates α -Syn with histidine tag attached to its N-terminal end. The only difference between the two His-tagged proteins is the number of amino acids in the leading sequence located between the histidine tag and the protein sequence, which are 10 amino acids longer for pTrc His-tagged α -Syn. Differences in the length and composition of this intermediate sequence may alter protein structure and may interfere with protein-protein interaction required for aggregation. Hence, this difference could underlie the relatively faster aggregation of WT α -Syn produced via pET15b vector compared to WT α -Syn of pTrc vector. Nevertheless, the use of His-tagged protein was one of the limitations in this study. The presence of the histidine tag in α -Syn sequence may cause a change in the way the protein folds to form aggregates or fibrils, which may also explain the faster aggregation observed for His-tagged α -Syn compared to untagged α -Syn. Therefore, this presumed structural change might be responsible for the faster aggregation observed for His-tagged α -Syn compared to untagged α -Syn. In addition, the presence of the histidine tag may result in some level of metal binding during different steps of purification. However, care was taken to use chelex-treated buffers and solutions during the course of experiment to eliminate the presence of any metal traces.

Optimisation of assay conditions was one of the main aims to start with. Since α -Syn is an acidic protein with a pI value of 4.7, therefore the lower the pH the more prone α -Syn will be to aggregate (Hoyer, Antony et al. 2002). Optimisation tests demonstrated that pH 7.4 fitted a preferable aggregation pattern than pH 6.5 did. Both Tris and Sodium phosphate (Na_2PO_4) were used in pH tests and other optimisation experiments. Although, Tris buffer was reported to be able to

interact with copper (Smith and Martell 1987), data was still considered as a control was always present and used in all experiments. In addition, other published studies have presented related work of α -Syn interaction with metals using Tris buffer (Uversky, Li et al. 2001b; Bharathi and Rao 2007). Various factors can affect the reproducibility of the results. Protein concentration is a critical factor that can significantly affect assay results. Variability seen with protein concentrations may originate from the different stages of protein preparation process, since it can influence the final protein concentration obtained at the end of the process. Some examples can be addressed such as freezing or not freezing bacterial cell pellets prior to purification. This could affect how well the bacterial cell wall is broken to release proteins. Also, it is important to consider the freshness of chemicals used throughout the process and maintaining the desired temperature when appropriate. In addition, protein concentration estimation method was another source of confusion that could effectively influence results. α -Syn concentration was estimated using Bradford assay throughout the project. When comparing values to those obtained by measuring concentration using absorbance at 275 nm, Bradford assay was found to underestimate protein concentration by approximately 10-fold. This is due to the lack of tertiary structure of α -Syn and the lack of enough ionisable amino acids on α -Syn to bind non-covalently to the dye via van der Waals forces. Thus, forming fewer complexes that are needed to measure the protein concentration. Estimating α -Syn concentration with absorbance at 275nm is reliable since the protein has aromatic amino acids (Tyrosine and Phenylalanine). However, this variation was not of an issue regarding to measuring protein concentration since it was taken into account while performing ThT assays. In addition, during experimental course, a different plate reader was used to perform assays and measure ThT fluorescence. Therefore, a sensitivity factor in detecting the fluorescence could add more into the differences obtained in the aggregation pattern when plotting the recorded ThT intensity values. ThT dye binding to the protein and the β -sheet structures can also contribute to the effectiveness of the results and assay accuracy. Yet, it is still a less controllable factor. Some of this

variability could be eliminated via the production of α -Syn batch that is enough to perform three repeats of the experiment. The next step then could be producing another batch of the protein and use it for the same purpose and compare results. This procedure may reduce the interference of protein preparation factor in assay results.

Metals in this study were used at 100 μ M which is low compared to other studies that have reported a similar effect of metals in accelerating α -Syn aggregation using concentrations of up to 2mM (Paik, Shin et al. 1999; Uversky, Li et al. 2001b). ThT assays focused mainly on three metals including copper, iron and aluminium. Results generated through this work regarding metals' effect on WT α -Syn brought no new findings. Copper, iron and aluminium to some extent all showed acceleration of α -Syn aggregation in ThT assays. While experiments performed with His-tagged α -Syn pointed to copper as the most effective metal in inducing α -Syn aggregation, experiments with untagged α -Syn demonstrated a higher effect for ferric iron. Such variation was present and has been reported in published studies where copper was most effective at enhancing α -Syn aggregation (Uversky, Li et al. 2001b; Rasia, Bertoncini et al. 2005; Binolfi, Rasia et al. 2006). On the other hand, previously aluminium was shown to cause rapid aggregation and high level of fluorescence of α -Syn compared to copper (Uversky, Li et al. 2001b). This variation could be a result of the different experimental conditions between these studies since different protein (35 μ M to 100 μ M) and metal concentration (100 μ M to 2mM), buffer and pH values (Mes buffer pH6.5 or NaP pH7.5) were used.

Importantly, potential metal binding sites in α -Syn were deleted in an attempt to assess the role of metal binding in α -Syn aggregation. All metals enhanced the aggregation of α -Syn metal binding mutants. Both N-terminal mutants (Δ 1-9 α -Syn, Δ 1-9 H50A α -Syn) showed similar results to WT α -Syn with regard to

accelerated aggregation with metals, mainly copper and iron. Moreover, copper was effective in inducing acceleration of the aggregation of H50A α -Syn. This could be explained by a possible coordination of copper by other sites, even in the absence of His 50 which reduces the competition to bind copper, and makes the protein prone to aggregate. In addition, mutating His 50 (being a copper interacting residue) to alanine was shown not to affect copper binding to the protein (Sung, Rospigliosi et al. 2006) indicating that copper binding sites are not dependent on each other. The effect observed of metals on α -Syn aggregation could be a result of metal binding to the C-terminus or the presence of the histidine tag within the N-terminal domain of his-tagged proteins. In addition, imidazole moieties of histidine residues represent a target for direct interaction with copper because they represent a high affinity copper binding element. Moreover, as ThT fluorescence indicates the presence of β -sheet rich fibrillar structures, therefore, WT α -Syn and its N-terminal mutants resulted in fibril formation, and metals accelerated the process. This was reported for WT α -Syn but no such data has previously been established for metal binding mutants (Uversky, Li et al. 2001b; Binolfi, Rasia et al. 2006). All proteins used in ThT assays aggregated and had increased ThT fluorescence. As ThT fluorescence was suggested to be a result of the interaction of ThT molecules within grooves between solvent-exposed side-chains of the amyloid fibrils (Hawe, Sutter et al. 2008) and to be enhanced by its specific binding to amyloid fibrils (Krebs, Bromley et al. 2005). Structurally, all proteins used in this study have the NAC domain, specifically residues 71-82, which was shown to be essential for protein aggregation (Uversky and Fink 2002). It has been shown in another study that fibrils formed by a truncated form of α -Syn (30-140) are similar in morphology and core size to those formed by the full length α -Syn (Qin, Hu et al. 2007). They reported that residues 32-102 constitute the core of α -Syn fibrils, which are present in WT, Δ 1-9 α -Syn, Δ 1-9 H50A α -Syn and H50A α -Syn used in this work. Hence, all mutants have the ability to aggregate and form fibrils.

The ability of copper to induce α -Syn aggregated was further supported by the cell culture model, where increased protein aggregates and not fibrils were detected via western blotting analysis. However, this method is capable of detecting large aggregates but not fibrils that are resistant to the denaturing conditions of the gel, since they would not pass through the polyacrylamide matrix pores which are not more than 200nm in diameter (Stellwagen 1998). Oligomeric aggregates of α -Syn were seen in samples representing all conditions, which imply the presence of a background level of oligomeric α -Syn in all cells over-expressing any form of α -Syn. It also could be related to the technique used to prepare the samples from cell lysates, which necessarily concentrate the protein and therefore generating some amount of α -Syn oligomers not present in cells. Even though, the latter can not be ruled out, it does not detract from or diminish the effect observed with copper where it altered the level of aggregates as demonstrated with western blotting. No role for iron in increasing aggregation was found in this work which is in disagreement of a previous study (Ostrerova-Golts, Petrucelli et al. 2000). In addition, intracellular aggregation of α -Syn in response to copper was shown to require both the N- and C-terminals, and deletion of either resulted in a clear loss of aggregation. It has been previously suggested (Brown 2009) that copper binding to α -Syn involves interaction between both domains; hence deletion of one domain would disturb copper binding especially that binding copper could occur via interaction of both domains of one α -Syn molecule. Moreover, results shown in this work did not include a comparison of the relative level of oligomer formation between cells expressing WT α -Syn and its disease-associated mutants in the absence of copper treatment which could be different. However, it has been reported that these mutants cause an increase formation of aggregated including fibrils (Li, Uversky et al. 2001).

In summary, all synucleins used in this study aggregated in the presence and the absence of metals. Metals at low, physiologically relevant concentrations induced

faster aggregation as demonstrated by ThT assay. Different assay conditions such as protein concentration, pH and even protein purification method and fluorescence detection method contribute to the final result. Mutating α -Syn at the N-terminal did not affect protein aggregation or prevent metal effect as shown by ThT assays. While deleting the C-terminus affected protein aggregation and blocked any obvious effect of copper on the protein aggregate formation as demonstrated by cell model but not *in vitro*. Copper increased α -Syn oligomers formation in a cell model. However, more repeats of these experiments under the same conditions are required to state a more clear result. Moreover, electron microscopy (EM) analysis would be of great beneficial to add more information about aggregation and fibrillation process of α -Syn metal binding mutants. Further ITC experiments of metal binding mutants of α -Syn would be beneficial to assess iron binding to these different mutant proteins. Also, it is important to consider complications accompanied with the method used to assess α -Syn aggregation. As well as the lack of reproducibility found while conducting the experiments.

Chapter Five: Regulation of Synuclein Expression

5.1 Introduction

The majority of Parkinson's disease (PD) cases are sporadic; however a subset of PD cases is inherited as a familial trait (autosomal-dominant trait). The α -Syn gene (SNCA) was one of the first genes implicated in PD aetiology, as the causative gene in familial PD, with the identification of point mutations in cases of early onset familial PD (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998). Additionally, α -Syn was shown to be the main component in LBs and LNs (Spillantini, Schmidt et al. 1997), even in brains of patients who do not carry α -Syn mutations (Mezey, Dehejia et al. 1998). Therefore, other mechanisms underlie the contribution of α -Syn in PD. Such mechanisms might include post-translational modifications, damage to the protein (oxidation), altered regulation of protein expression or protein degradation (Chiba-Falek and Nussbaum 2001). The importance of assessing the regulation of α -Syn expression comes from other findings, such as mice over-expressing α -Syn have been found to display neuronal inclusions similar to LBs, increased degradation of dopaminergic neurons and motor deficits (Masliah, Rockenstein et al. 2000). Moreover, genomic multiplications in SNCA were considered to be risk factors for PD (Singleton, Farrer et al. 2003) and polymorphic alleles in synuclein promoter were found to be associated with sporadic PD (Kruger, Vieira-Saecker et al. 1999).

Little is known about the regulation of α -Syn expression. Many studies have suggested that the genomic multiplications in SNCA lead to increased gene expression (Singleton, Farrer et al. 2003; Miller, Hague et al. 2004; Pals, Lincoln

et al. 2004) and cause excess amounts of α -Syn, which might be sufficient to increase susceptibility to familial PD (Chiba-Falek, Kowalak et al. 2005). In addition, increased levels of α -Syn mRNA in substantia nigra (SNpc) neurons from patients with idiopathic PD compared to controls have been reported, which emphasizes the importance of normal regulation of α -Syn expression (Chiba-Falek, Lopez et al. 2006; Grundemann, Schlaudraff et al. 2008). Many studies have aimed to characterize the promoter (enhancer) elements in SNCA gene and to analyse the potential sites for transcription factors, particularly in the region upstream of SNCA gene (Chiba-Falek and Nussbaum 2001). Others attempted to investigate the association between promoter polymorphisms of SNCA gene and sporadic PD (Pals, Lincoln et al. 2004; Mueller, Fuchs et al. 2005). The most studied genetic variation was the REP1 polymorphism, which has been shown to have a direct relevance to PD by affecting gene expression and age of disease onset based on the allele size (Chiba-Falek and Nussbaum 2001; Maraganore, de Andrade et al. 2006; Kay, Factor et al. 2008). Polymorphisms at this complex dinucleotide repeat NACP-REP1, located ~10kb upstream of the SNCA transcriptional start site, have gained attention as they have been associated with sporadic PD (Kruger, Vieira-Saecker et al. 1999; Linnertz, Saucier et al. 2009). This region has been suggested to likely contain the gene promoter and putative regulatory sequences, and to influence the expression of α -Syn (Touchman, Dehejia et al. 2001). Moreover, the ~10kb sequence upstream of SNCA gene, including the NACP-REP1, has been shown to be necessary for normal expression (Chiba-Falek and Nussbaum 2001; Touchman, Dehejia et al. 2001) and for regulating the transcriptional activity of SNCA gene. SNCA activity has also been found to be modulated by the length of the REP1 allele (Chiba-Falek, Touchman et al. 2003). Assessment of promoter activity of deletion constructs of that sequence confirmed its importance in controlling α -Syn expression. It has been shown that the absence of an 880bp segment containing the NACP-REP1 repeat led to total elimination of the promoter activity in neuroblastoma cell line (SHSY-5Y) (Chiba-Falek and Nussbaum 2001).

A role for β -Syn, which shares structural homology and sub-cellular localisation pattern similar to α -Syn, in modulating the pathology of α -Syn has been suggested. It has been shown that β -Syn can inhibit the aggregation process of α -Syn *in vitro* (Uversky, Li et al. 2002b) and can reduce its toxicity in animal models *in vivo* (Hashimoto, Rockenstein et al. 2001). Importantly, it has been reported that levels of α -Syn mRNA increases and β -Syn mRNA decreases in SNpc of PD brains compared to controls (Rockenstein, Hansen et al. 2001) which is the opposite to normal brains aging (Li, Lesuisse et al. 2004). Decreased levels of β -Syn mRNA, as demonstrated by RT-PCR, have also been reported in brains of patients of dementia with Lewy bodies (Beyer, Domingo-Sabat et al. 2010). These observations emphasize the importance of a balanced regulation of both α -Syn and β -Syn, and suggest that β -Syn might be a natural negative regulator of α -Syn. Information about regulators of β -Syn expression had not been reported at the start of this study.

Metals, as repeatedly mentioned, were linked to PD as risk-increasing factors and were shown to induce α -Syn aggregation *in vitro* (Uversky, Li et al. 2001b). Although the cellular function of α -Syn is not known, however, giving that α -Syn binds metals (Cu^{2+} and Fe^{3+}) (Binolfi, Rasia et al. 2006) and the increased iron content in PD brains (Dexter, Wells et al. 1989; Dexter, Carayon et al. 1991) where aggregated α -Syn constitutes a major component of Lewy bodies (Spillantini, Schmidt et al. 1997), it all emphasizes the role played by metals in PD. It has been shown that expression of prion protein (PrP), involved in Prion disease pathology, was shown to be regulated by copper (Toni, Massimino et al. 2005; Varela-Nallar, Toledo et al. 2006b) via metal response element (MRE's) sequences, and mediated by metal-responsive transcription factors such as MTF-1 (Bellingham, Coleman et al. 2009). Metal responsive transcription factor-1 (MTF-1) can bind to MRE sequences and regulate metallothionein genes in response to metals such as zinc (Stuart, Searle et al. 1984; Radtke, Heuchel et

al. 1993). Moreover, SP1, a general activator of transcription, can also bind to putative MRE sites (Ogra, Suzuki et al. 2001) and was shown to mediate copper regulation of PrP expression with MTF-1 (Bellingham, Coleman et al. 2009). Therefore, it could be hypothesized that metals, among different factors and conditions, could cause altered regulation of α -Syn expression.

In this study, human neuroblastoma cells SHSY-5Y, were used. Promoter analysis was performed largely based on the finding of MRE's in α -Syn promoter, as well as β -Syn promoter. Therefore, it is essential to determine the potential involvement of MRE's in α -Syn promoter activity and to assess any changes in expression in response to metal exposure. A reporter assay, utilizing plasmid vector (pGL3basic) containing synuclein promoter fragments, was used in the following experiments to assess synuclein's promoter activity. Quantitative real time PCR is a widely used method to assess transcriptional activation of genes in response to different treatments or conditions, however, a reporter based assay was chosen as a start point in this work.

5.2 Synuclein Promoter Analyses

For the assessment of synuclein promoter activity, synuclein promoter fragments were cloned into Luciferase reporter constructs and transfected into SHSY-5Y cells. The 10.8kb region upstream of the synuclein transcriptional start site was divided into smaller DNA segments and cloned into the reporter assay plasmid vector (pGL3basic). Promoter fragments were gradually shortened to further identify the regulatory regions of the synuclein promoters with and without metal exposure.

5.2.1 Luciferase Based Analysis of Synuclein Promoter

Fragments of both human α -Syn and β -Syn promoters were cloned and inserted into pGL3basic reporter vector to enable assessment of their activities. SHSY-5Y cells were transfected with two individual reporter vectors at the same time. The first is the experimental vector (pGL3basic) in which the promoter sequence of interest is cloned upstream of the Firefly Luciferase gene. The second is the control vector, Renilla Luciferase, which serves as internal control for the transfection. The activity of both Luciferases was detected in the same sample using the Dual-Luciferase Reporter Assay System (Promega). The activity of the experimental reporter was normalized to the activity of the control reporter to minimize experimental variability caused by differences in cell viability and transfection efficiency. During the course of experiments, assessing synucleins promoter's activity, two internal control vectors were used pRL-SV40 and pRL-TK. The difference between both vectors is that pRL-SV40 contains the SV40 enhancer and early promoter elements which provides a high level of expression of Renilla Luciferase in the co-transfected mammalian cells, while pRL-TK contains the herpes simplex virus thymidine kinase (HSV-TK) promoter which produces a low to moderate expression level. Results obtained using both vectors were similar.

5.2.1.1 Alpha Synuclein Promoter Activity Assessment

To evaluate the promoter activity of α -Syn, the -10.8kb region was divided into smaller segments and cloned into pGL3basic reporter plasmid. Numbers such as (-10.8/-6) refer to kilobases upstream of the transcriptional start site. Initial analysis was performed on fragments (α -Syn-10.8/-6, α -Syn-6/-1.3, α -Syn-3.6/-1.3, α -Syn-1.3/1 and α -Syn-4.1/1). Selection of promoter fragments for potential activity was based on the presence of MRE's in their sequence to which transcription factors such as MTF-1 and SP1 can bind.

Figure 5.1 shows that intron1 sequence of α -Syn is included as it has been reported to be an important regulatory region and cooperates with region 5' to exon1 in order to mediate gene transcription (Clough and Stefanis 2007; Clough, Dermentzaki et al. 2011).

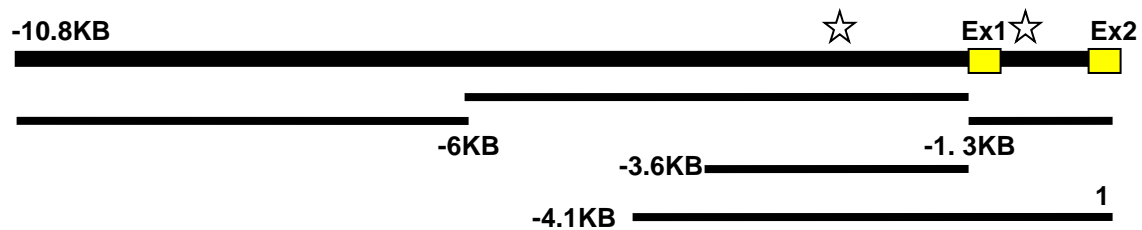


Fig. 5.1 Schematic illustration of the 10.8kb region upstream of α -Syn transcriptional start site. Stars represent transcription factor binding sites (MTF-1), metal response elements MRE's, as identified using Transcriptional Element Search Software (TESS) (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>).

α -Syn promoter analysis revealed two MRE's, (TGCGCTC and GTGTGCA), contained in α -Syn-3.6/-1.3, α -Syn-1.3/1 and α -Syn-4.1/1 fragments tested in this project, and located at -1182bp and -2517bp upstream of the transcriptional start site.

Results showed an increase in the activity of promoter fragment α -Syn-6/-1.3 compared to α -Syn-10.8/-6. Higher activity was observed with α -Syn-3.6/-1.3 fragment and α -Syn-4.1/1 compared to other fragments (Figure 5.2).

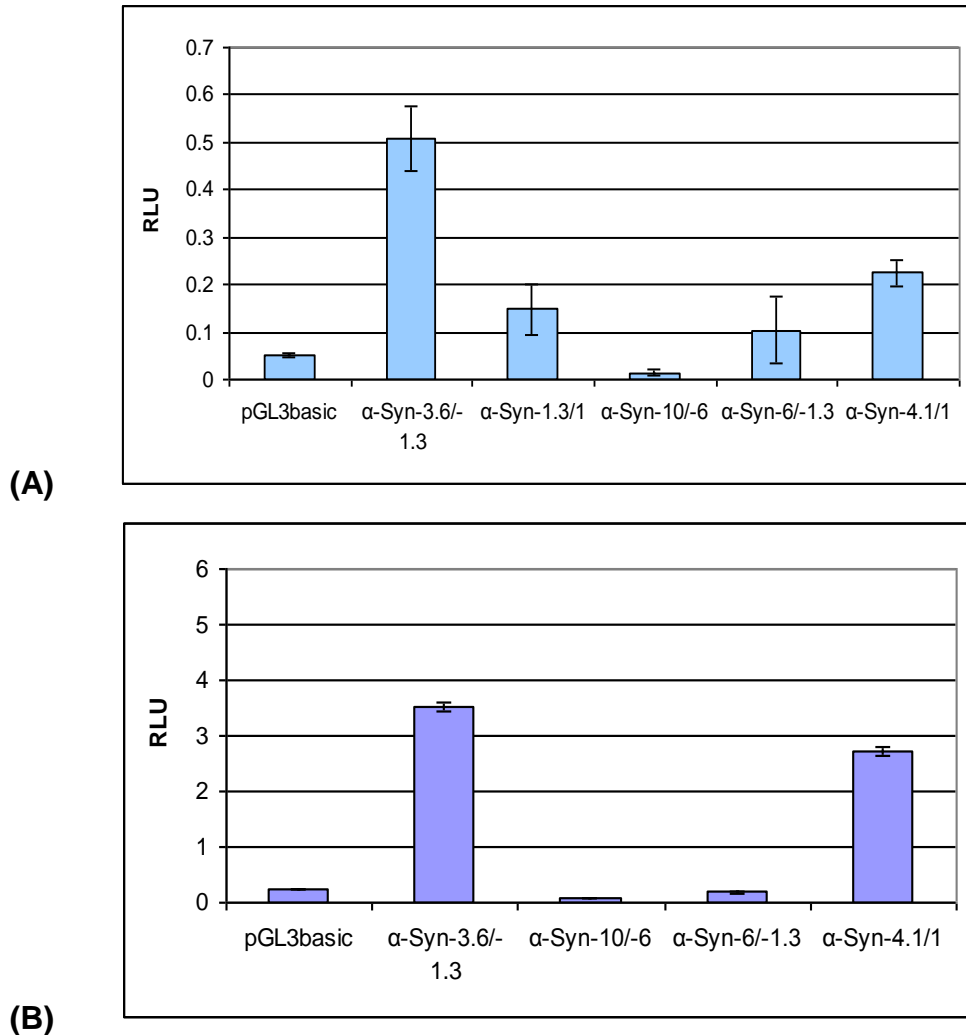


Fig. 5.2 Relative Luciferase activity of different α -Syn promoter fragments in the absence of metals. SHSY-5Y cells were grown in normal media and transfected with 200ng of pGL3basic vector containing one of the promoter fragments with 10ng of the control vector (A) pRL-SV40 and (B) pRL-TK. Error bars represent standard error of four and three independent experiments, respectively. RLU stands for: Relative Luciferase Units.

α -Syn-3.6/-1.3 and α -Syn-4.1/1 showed more activity than other fragments tested when co-transfected with either pRL-SV40 or pRL-TK controls. On the other hand, α -Syn-10.8/-6 showed no activity comparing to the other fragments and it was as low as the control (pGL3basic vector) that has no DNA insert in it. Results

shown in Figure 5.2 reflect transfections performed on cells grown in normal growth media (DMEM + Ham's F-12).

5.2.1.2 Effect of Metals on Alpha Synuclein Promoter Activity

SHSY-5Y cells were grown in normal media (DMEM + Ham's F-12) until they reached 60-80% density and then transfected with 200ng of each of the promoter fragments along with 10ng of the control vector (pRL-TK). Metal treatment was performed ~16 hours past transfection with fresh media. Metal (Cu^{2+} and Fe^{3+}) were added at either 10 μM or 100 μM final concentrations and cells were incubated further for ~30 hours (Figure 5.3).

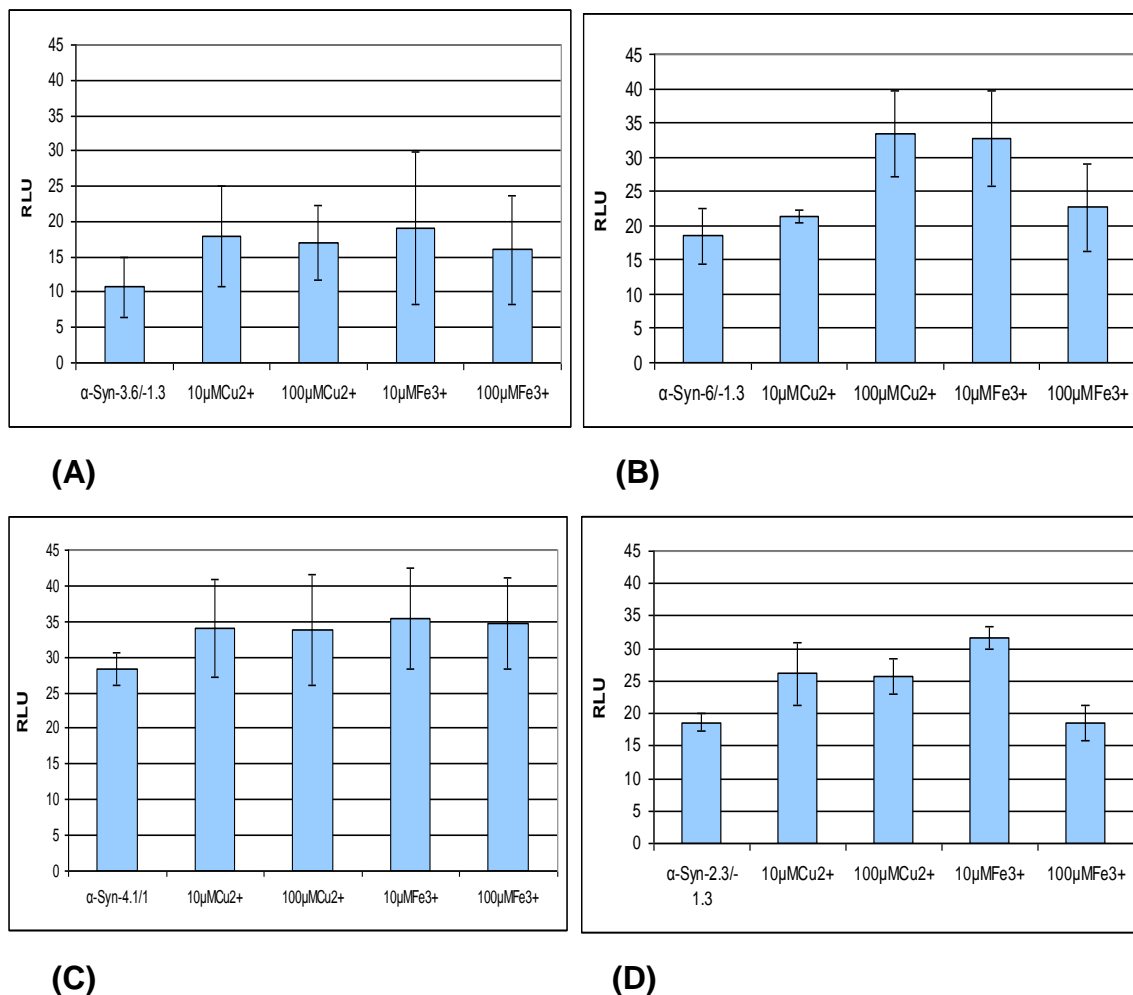


Fig. 5.3 Relative Luciferase activity of different α -Syn promoter fragments in the presence of metals. The effect of the presence of 10 μ M or 100 μ M of Cu²⁺ or Fe³⁺ on α -Syn promoter fragments (A) α -Syn-3.6/-1.3 (B) α -Syn-6/-1.3 (C) α -Syn-4.1/1 and (D) α -Syn-2.3/-1.3. SHSY-5Y cells were grown in normal media and co-transfected with pRL-TK as control vector. Error bars represent standard error of the mean of three experiments. Statistical analysis was performed using Student-t test and was found to be above the $p < 0.05$ threshold.

Results in Figure 5.3 show that metals have little to no effect on increasing the activity of α -Syn promoter fragments tested either containing MRE's (α -Syn-3.6/-1.3, α -Syn-1.3/1 and α -Syn-4.1/1) or not containing any MRE's (α -Syn-2.3/-1.3). This could be due to performing this set of experiments in normal media (DMEM

+ Ham's F-12) which contains trace levels of metals that could make observing any differences in metal regulation of promoter activity difficult. Promoter fragment α -Syn-10/-6 was not assessed with metals as it showed no activity comparing to other fragments. Therefore, it was important to assess whether the presence of metals in the media would influence promoter activity. This is an important precursor to experiments which continue to assess the effect of metals on promoter activity, whereby metal treatment was performed after removing the normal media and replacing it with chelex-treated media. Therefore, after transfecting cells in normal media (DMEM + Ham's F-12), basal activity of α -Syn promoter fragments was not affected by adding fresh chelex-treated media. Basal activity of α -Syn-3.6/-1.3 fragment shown in Figure 5.4 is an example of the effect of changing the media after transfection (Figure 5.4).

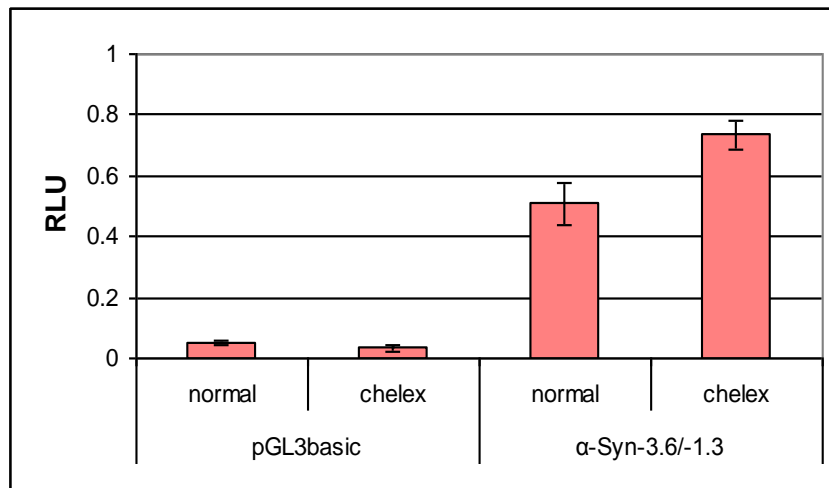
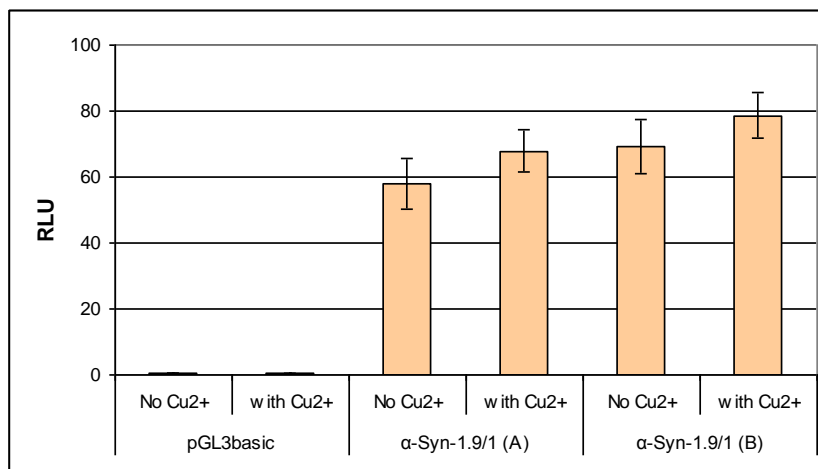


Fig. 5.4 Relative Luciferase activity of α -Syn-3.6/-1.3 fragment in two different conditions. α -Syn-3.6/-1.3 was transfected into SHSY-5Y cells grown in two different conditions, normal and chelex-treated media, after being transfected in normal media. Cells transfected with pGL3basic vector with no α -Syn fragment was used as a control. Error bars represent standard error of the mean of three experiments.

Figure 5.4 above shows that activity of α -Syn-3.6/-1.3 fragment was slightly higher when SHSY-5Y cells were cultured in chelex-treated media, from which metal traces has been removed, compared to those left in normal media. No activity was shown in both conditions with cells transfected with pGL3basic control with no insert in it. This result suggests that changing the media in which cells were grown after transfection into a media from which metals are removed could affect promoter activity. A similar result was obtained when such assessment was repeated and accompanied with metal (copper) treatment. The promoter fragment chosen for this experiment was α -Syn-1.9/1 because it showed activity considerably higher than the control and because it was available at time of the experiment. SHSY-5Y cells were grown in normal media until reaching 60-80% density, and then cells were transfected with a promoter fragment (α -Syn-1.9/1). Copper treatment and changing the media was performed together, ~16hr after transfection, keeping half the cells in normal media and changing the media into chelex-treated media for the other half. Both groups of cells were harvested ~30hrs after metal treatment, lysed and analysed (Figure 5.5, A). In addition, a third set of SHSY-5Y cells were grown in normal media until the proper density (60-80%), then the media was changed into chelex-treated media prior to transfection and metals were added to the cells in the same media (chelex-treated). Transfecting cells with the later condition showed major effect on transfection efficiency and much less activity compared to other condition used and described above (Figure 5.5, B).

[A]



[B]

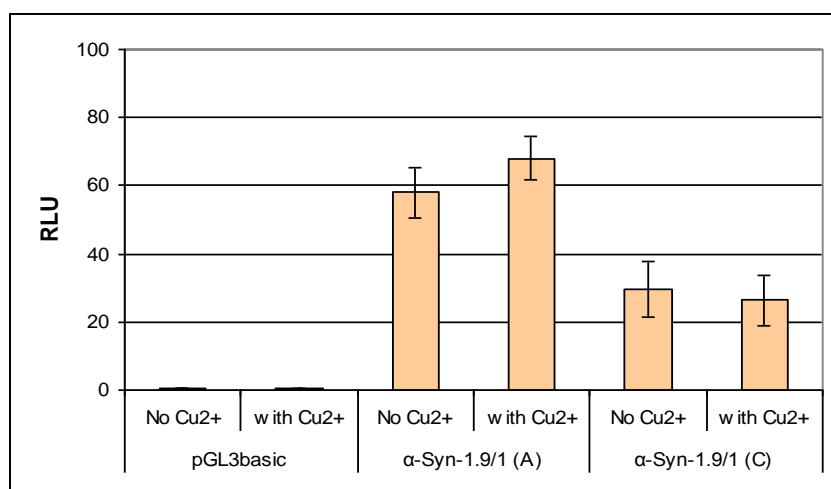


Fig. 5.5 Relative Luciferase activity of α -Syn-1.9/1 promoter fragment under different conditions where [A] SHSY-5Y cells were transfected in normal media and treated with Cu²⁺ in (A) normal media, and (B) after changing the media into chelex-treated media. [B] SHSY-5Y cells were transfected in (A) normal media (C) chelex-treated media and then treated with Cu²⁺ in the same media. Error bars represent standard error of the mean of three experiments.

Results in Figure 5.5 above show and confirm that the effect of metal treatment on promoter activity was not influenced by changing the media in which the cells are incubated after transfection, when cells were transfected in normal media. Also results in Figure 5.5, B shows considerable decrease in the activity of α -Syn promoter fragment when transfection was performed in chelex-treated media and

followed by metal treatment. Therefore, the conditions utilised in Figure 5.5 A, rather than 5.5 B were used in the assessment of metals' effect on α -Syn and β -Syn promoter activity such as that presented in Figure 5.6, as well as the subsequent assessments.

A further set of experiments was conducted to assess activity of α -Syn promoter fragments utilising the procedure described earlier, with pRL-SV40 as the internal control vector and metals were added to transfected cells in chelex-treated media. SHSY-5Y cells were grown in normal media until 60-80% density was reached and then transfected with 200ng of each of the promoter fragments with 10ng of the control vector (pRL-SV40). Metal treatment was performed ~16hrs after transfection just after the media was changed into fresh chelex-treated media. Metals were added at 1, 10, 100 μ M final concentrations and cells were incubated for further ~30hrs. Similar to metals treatment in normal media (Figure 5.6), metals showed no statistically significant effects on α -Syn promoter activity.

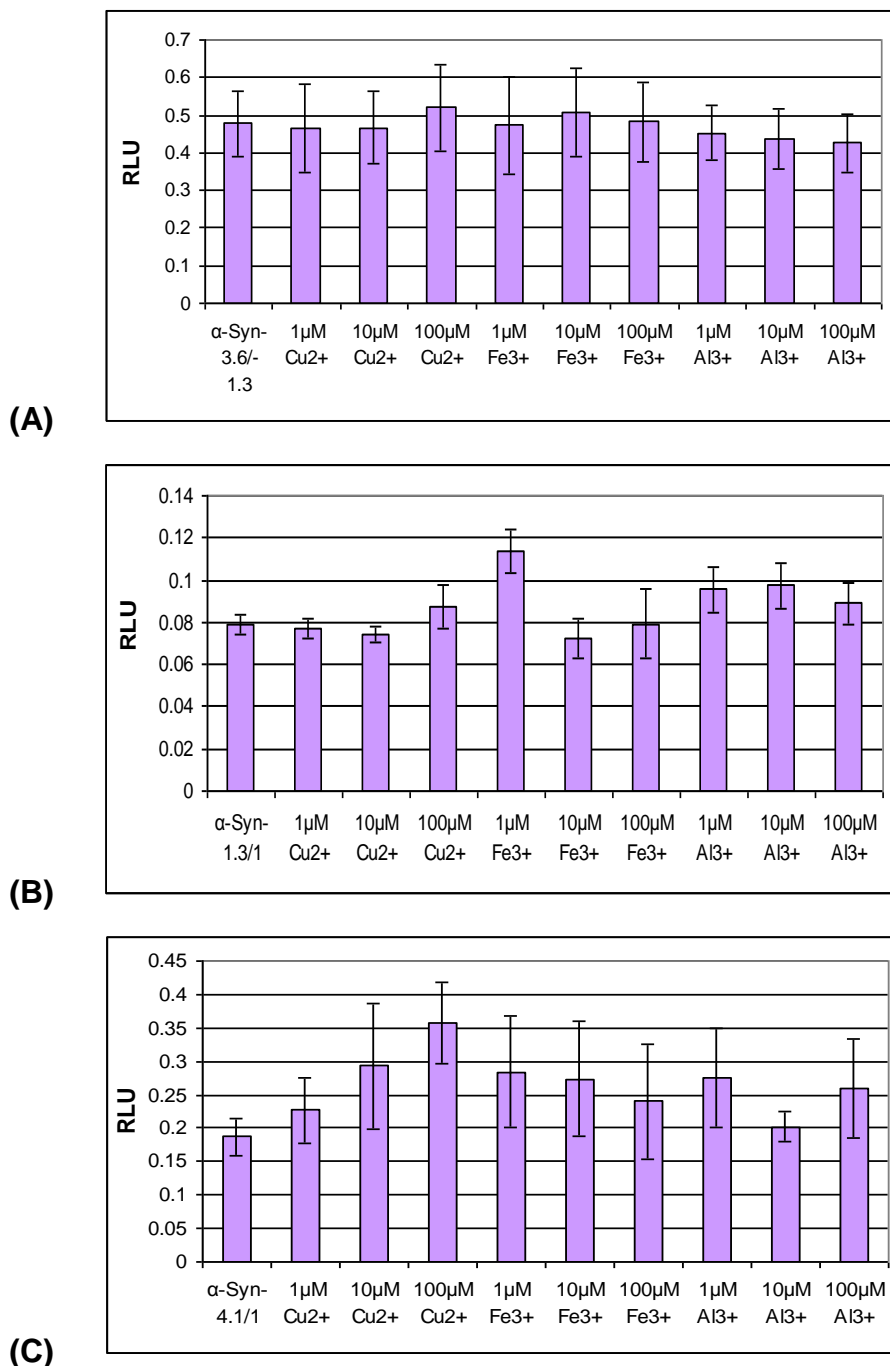


Fig. 5.6 Relative Luciferase activity showing the effect of metals on α -Syn promoter fragments. 1, 10, 100 μ M of metals (Cu^{2+} , Fe^{3+} , Al^{3+}) were added on SHSY-5Y cells transfected with α -Syn promoter fragments (A) α -Syn-3.6/-1.3 (B) α -Syn-1.3/1 and (C) α -Syn-4.1/1. SHSY-5Y cells were grown in normal media and co-transfected with pRL-SV40 as internal control vector. Metal treatment was performed in chelex-treated media. Error bars represent standard error of the mean of four experiments.

Results presented in Figure 5.6 show little effect of metals tested on increasing activity of α -Syn promoter fragments. The effect shown with $1\mu\text{M Fe}^{3+}$ on α -Syn-1.3/1 and with $100\mu\text{M Cu}^{2+}$ on α -Syn-4.1/1 was not significant as demonstrated by Student's t-test. Additionally, there was little difference between results shown with α -Syn fragments (α -Syn-3.1/-1.3, α -Syn-4.1/1) when treated with metals in either normal or chelex-treated media.

5.2.1.3 Beta Synuclein Promoter Activity Assessment

Similar to α -Syn, the β -Syn promoter was cloned into pGL3basic plasmid vector to be assessed for its potential activity (Figure 5.7).

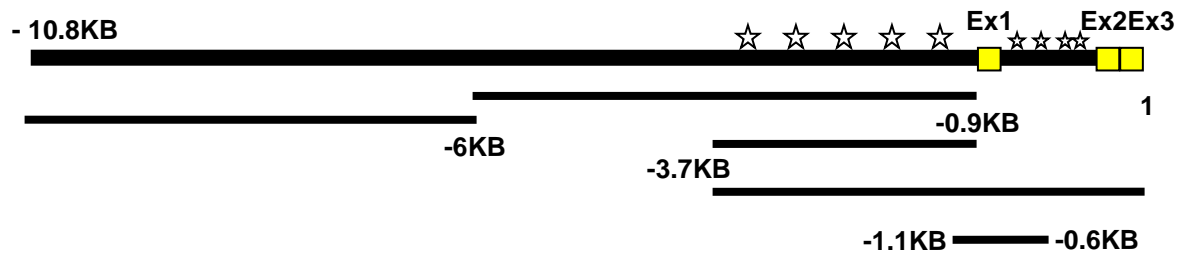


Fig. 5.7 Schematic illustration of the 10.8kb region upstream of β -Syn transcriptional start site. Stars represent transcription factor binding site (MTF-1), metal response elements MRE's, as identified using Transcriptional Element Search Software (TESS) (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>).

Basal activity of selected β -Syn fragments were assessed using the Luciferase based assay. The selection of β -Syn fragments was based on location similarity to those of α -Syn fragments and taking in consideration the presence of MRE's in that region. Analysis of the β -Syn promoter fragments using Transcriptional

Element Search Software (TESS) (McHugh, Wright et al. 2011; Schug 2008) revealed the presence of more MRE's in β -Syn promoter compared to α -Syn promoter (Figure 5.1). SHSY-5Y cells were grown in normal media (DMEM + Ham's F-12) until they reached 60-80% density and then transfected with 200ng of each of the promoter fragments inserted in pGL3basic vector along with 10ng of the internal control vector (pRL-TK). Cells were then incubated for ~48hr after transfection and then harvested for analysis (Figure 5.8).

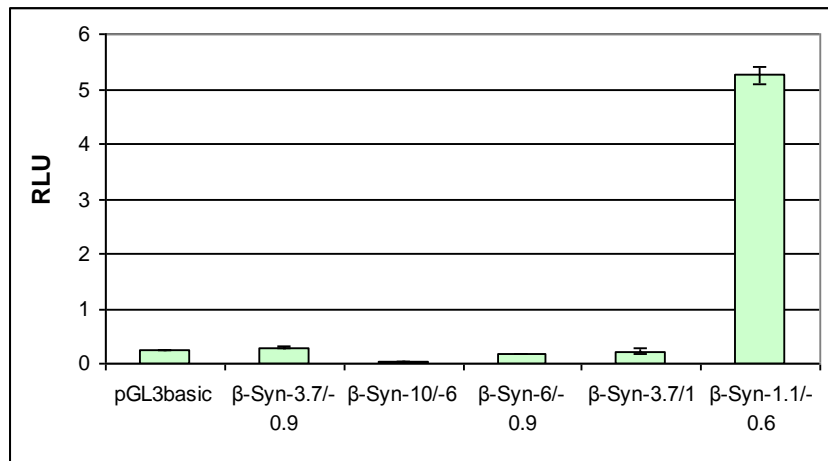


Fig. 5.8 Relative Luciferase activity of different β -Syn promoter fragments in the absence of metals. SHSY-5Y cells were grown in normal media and transfected with 200ng of pGL3basic vector containing one of the promoter fragments with 10ng of the control vector (pRL-TK). Error bars represent standard error of the mean of three experiments.

β -Syn promoter fragments showed much lower activity compared to α -Syn and also close to the background activity shown by the empty vector (pGL3basic with no promoter insert in it) when either internal control is present (pRL-SV40 or pRL-TK). β -Syn-1.1/-0.6 fragment showed the highest activity level among all fragments tested which was recently reported (McHugh, Wright et al. 2011).

5.2.1.4 Effect of Metals on Beta Synuclein Promoter Activity

Similar to the previously conducted assessment of α -Syn promoter fragments, SHSY-5Y cells transfected with a β -Syn promoter fragment, were treated with metals (Cu^{2+} , Fe^{3+} and Al^{3+}) to assess whether metals cause any changes to their activities. SHSY-5Y Cells were grown in normal media until 60-80% density and then transfected with 200ng pGL3basic with a β -Syn promoter fragment inserted in it along with 10ng pRL-TK as an internal control for transfection. Metals (Cu^{2+} , Fe^{3+} and Al^{3+}) were added at 10 μM and 100 μM as final concentration ~16hrs after transfection. Cells were then incubated for another 30hrs and then harvested for analysis. This experiment was performed for two fragments of the β -Syn promoter (β -Syn-3.7/-0.9 and β -Syn-0.9/1) shown in Figure 5.9.

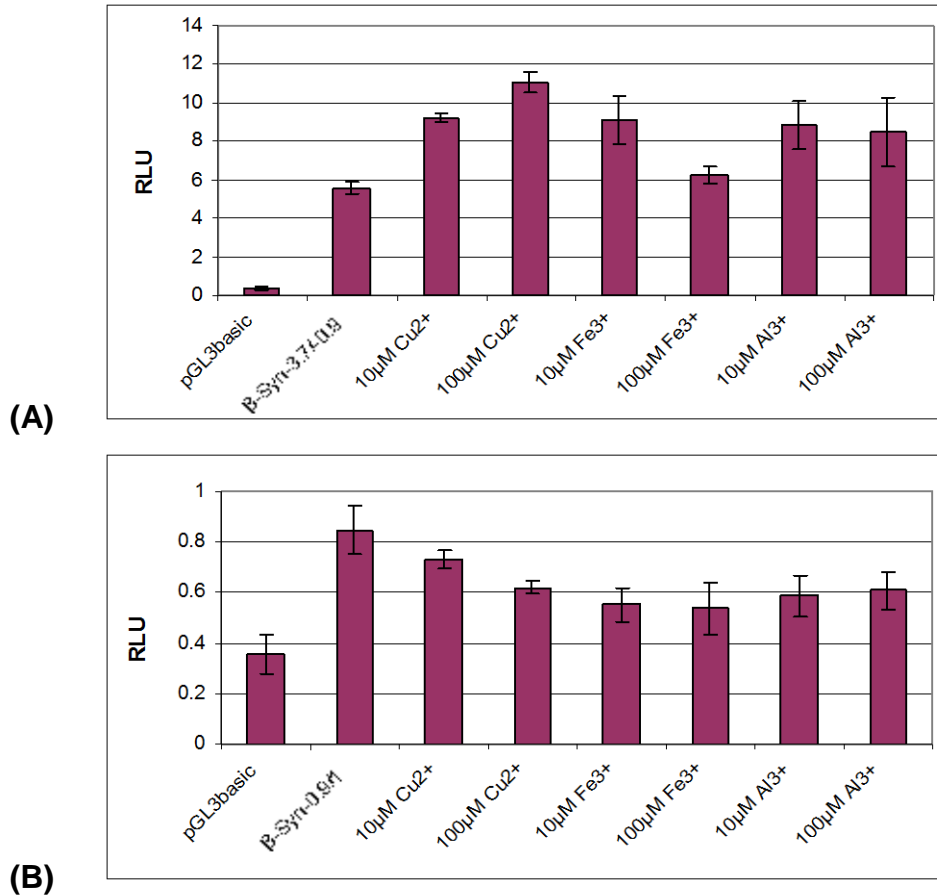


Fig. 5.9 Relative Luciferase activity of different β -Syn promoter fragments in the presence of metals. The effect of adding 10 μ M and 100 μ M of metals (Cu²⁺, Fe³⁺ and Al³⁺) on SHSY-5Y cells transfected with β -Syn promoter fragments (A) β -Syn-3.7/-0.9 and (B) β -Syn-0.9/1. SHSY-5Y cells were grown in normal media and co-transfected with pRL-TK as internal control. Error bars represent standard error of the mean of three experiments.

Metals resulted in no effect on the activity of β -Syn-0.9/1 fragment (Figure 5.9, B). However, copper at 10 μ M and 100 μ M showed a significant increase in promoter driven activity of β -Syn-3.7/-0.9 (as shown by Figure 5.9, A; Student-t test $p < 0.05$).

5.3 Discussion

The importance of understanding the regulation of α -Syn expression is clear from studies showing increased levels of α -Syn mRNA and protein in PD brains, in addition to the identification of several genomic multiplications of the α -Syn gene in families with PD and DLBD (Singleton, Farrer et al. 2003; Pals, Lincoln et al. 2004; Nishioka, Hayashi et al. 2006). Moreover, β -Syn has been presented as a negative regulator of α -Syn due to its ability to inhibit its aggregation and reduce its toxicity (Hashimoto, Rockenstein et al. 2001; Uversky, Li et al. 2002b; Hashimoto, Bar-On et al. 2004). This emphasized the need to have a strict regulation of both α -Syn and β -Syn and to identify regulators of their expression to assist avoiding disease progression. The assessment of the regulation of synuclein expression as reported throughout this project aimed to identify the regulatory regions in both α -Syn and β -Syn promoters, and to assess how factors, such as metals, can affect synuclein's expression. This consideration of metals as regulatory factors was supported by the *in vitro* findings of metals' enhancement of α -Syn aggregation (Uversky, Li et al. 2001b) and by copper's regulatory role reported for PrP in Prion disease (Toni, Massimino et al. 2005; Varela-Nallar, Toledo et al. 2006b).

The 10.8kb region upstream of SNCA transcriptional start site has been the main focus in research of the regulation of SNCA gene expression (Chiba-Falek and Nussbaum 2001). This is mainly due to the presence of polymorphisms at a complex repeat site approximately ~10kb upstream of SNCA gene which was accompanied with increased expression levels of α -Syn (Fuchs et al. 2008), even though the region -10/-6 fragment was shown to have low promoter activity (Chiba-Falek and Nussbaum 2001). Several constructs, covering varying distances from the transcriptional start site of SNCA gene, were created to perform a deletion analysis to this region in order to facilitate observing the ones with enhancer activity. These constructs included the first intronic sequence of

SNCA gene based on reports highlighting the importance of that region in regulating α -Syn transcription (Clough and Stefanis 2007; Jowaed, Schmitt et al. 2010). Response elements mediating α -Syn transcriptional regulation by growth factors have been characterized in intron1 of SNCA gene (Clough and Stefanis 2007). Moreover, expression-relevant GATA TF binding sites have been identified in intron1, and therefore they can play a role in regulating SNCA gene expression (Scherzer, Grass et al. 2008). In addition, promoter analyses and prediction software identified several transcription factor binding sites upstream from ATG in human SNCA gene (Jowaed, Schmitt et al. 2010). Thus including intron1 of SNCA gene was relevant to transcriptional regulation studies.

Assessment of short DNA segments of that promoter region (α -Syn-3.6/-1.3 and α -Syn-4.1/1) resulted in high enhancer activity (Figure 5.2). This result is consistent with another study indicating that this part (-6/-1.3kb) of the promoter conserves the enhancer ability to drive the expression (Chiba-Falek and Nussbaum 2001), especially that the part (-10.8/-6kb) showed no activity. It is also in agreement of their work as they referred to the importance of a 400pb segment upstream of exon1, being sufficient to drive the expression, as demonstrated via similar deletion analysis and Luciferase assay. This 400bp is contained within both constructs referred to earlier (α -Syn-3.6/-1.3 and α -Syn-4.1/1) but not in α -Syn-1.3/1 which showed less activity (Figure 5.2, A). On the other hand, analysis of β -Syn promoter size-matching fragments to those of α -Syn had activity that was either lower or slightly higher than that of the background which was different to activity levels seen with α -Syn. Although both α -Syn and β -Syn promoters possess a similar range of transcription factor binding sites in the region comprising (-6/Ex1) such as Sp1 and AP2 which would imply that they would have a similar activity, however the effect on regulation of transcription might be different. One fragment, β -Syn-1.1/-0.6, had a considerably high activity comparing to other closely located promoter fragments such as β -Syn-3.7/1 or β -Syn-3.7/-0.9, in human dopaminergic SHSY-5Y cells (Figure 5.8).

This could be due to the absence of repressors from this small fragment while present in others, and could suggest that promoter sequence of β -Syn might be overlapped with exon1.

The change in conditions reported throughout the assessment of synucleins promoter activity did not affect results and activity pattern. Such a change was the use of two different internal control vectors, pRL-SV40 or pRL-TK, and the level of activity of tested fragments was alike when either internal control plasmid was used. This established that the main purpose of using those controls was fulfilled which is to control transfection. Due to the low level of Firefly Luciferase expression driven by synuclein promoter fragments comparing to the high level of Renilla Luciferase expression driven by pRL-SV40 plasmid used initially, experiments were carried out afterwards using pRL-TK plasmid which has a moderate Luciferase expression and makes it more relevant. Putting transfected cells into chelex-treated media prior to metal treatment was another change in conditions which did not inhibit promoter activity. However, transfecting the cells in chelex-treated media resulted in a notable decrease in activity level (Figure 5.5, B). Chelex-treated media is the normal growth media (DMEM + Ham's F-12) treated with chelex beads for few hours to remove metal traces present in the media, as metal concentrations as low as 1 μ M were aimed to be tested. This could imply that metals are essential and their removal could cause decrease in transfection efficiency.

Metals have been linked to PD aetiology and were found to accelerate α -Syn aggregation *in vitro* (Uversky, Li et al. 2001b). Disturbed metal homeostasis has also been recognized in brain of patients with PD (Dexter, Wells et al. 1989). Moreover, analysis of α -Syn and β -Syn promoter sequence revealed the presence of MRE's, which are highly conserved motifs found in other genes' promoters that are regulated in response to metal exposure to enable metals

metabolism and detoxification (Lichtlen, Wang et al. 2001). This suggests that synuclein's may be regulated by transcription factors such as MTF-1. Thus, it was important to evaluate the role of metals and MRE's present in synuclein's promoter and whether they participate in regulating their expression. Transfections directed to assess metals' effect on promoter activity were not performed in chelex-treated media, hence maintaining transfection efficiency as high as possible. The use of either internal control plasmid vector (pRL-SV40 or pRL-TK) did not influence the tested effect of metals on promoter activity. Metals showed neither decrease nor a major increase of synucleins promoter activity as demonstrated by Luciferase expression. Results however carried some variability, where adding Fe^{3+} at 10 μM concentration resulted in a noteworthy increase in $\alpha\text{-Syn-2.3/-1.3}$ activity which is a construct with no MRE's. Moreover, adding Cu^{2+} at 10 μM and 100 μM concentration caused considerable increase in $\beta\text{-Syn-3.7/-0.9}$ activity (Figure 5.9, A). Bearing in mind that standard error for that set of experiments is small, in addition to the presence of MRE's within this fragment $\beta\text{-Syn-3.7/-0.9}$ as shown using Transcriptional Element Search Software (TESS); hence it is possible that this effect is not false. However, this does not imply that MRE's present in this construct are responsible for the observed effect, as this need to be confirmed by other methods such as EMSA (Electrophoretic Mobility Shift Assay) which determine the binding of a transcription factor to DNA (MRE's). In addition, copper's effect on regulating PrP expression was mediated by MLS sites that does not bind MTF-1 TF (Varela-Nallar, Toledo et al. 2006b), hence more work and assessments are required to confirm any seen effects. Recently, it has been shown that among $\beta\text{-Syn}$ constructs reported throughout the chapter, $\beta\text{-Syn-1.1/-0.6}$ which possesses the highest basal promoter activity and an MRE binding site is up-regulated in response to MTF-1 TF (McHugh, Wright et al. 2011).

The highest concentration of metals used in Luciferase assays was 100 μM which was found to cause no damage to the cells. It was reported that 200 μM Cu^{2+} is

the concentration required for induction of a stress response in cells (Murata, Gong et al. 1999; Varela-Nallar, Toledo et al. 2006b). The results obtained from the Luciferase assays are preliminary, since such assessment was not performed on a wide range and even on smaller constructs of synuclein promoters. Therefore, a link supporting a role for metals (such as Cu^{2+}) in regulating synuclein expression was not fully established resembling that of Cu^{2+} and Prp, APP or at the recently identified for β -Syn (McHugh, Wright et al. 2011). However, it appears that removing metals from the growth media leads to alterations in α -Syn expression (Figure 5.4) separate from being a result of a modification in transfection efficiency. In addition to the enhancing effect of Cu^{2+} observed on β -Syn-3.7/-0.9 fragment, it can be assumed that metals participate to some level in regulating synuclein expression. The Luciferase assays require more optimisation and further repeat, as results obtained had variability and were not sufficient to precisely determine metals' effect on synuclein regulation.

Chapter Six: General Discussion

Interaction between metals, such as copper and iron, with α -Syn was the main focus of this thesis. This interaction was addressed in three main aspects in which metals were relevant to the activity, aggregation and expression of α -Syn. Metals were required for α -Syn proposed ferrireductase activity and were also looked at as enhancers of α -Syn aggregation. In addition, an aspect of exploring whether metal exposure enhanced α -Syn expression was addressed.

A metal dependent activity for α -Syn was determined which is reducing iron using copper as a co-factor. Establishing the ability of α -Syn as ferrireductase required a series of complementary experiments. The start point was ITC experiments to demonstrate that α -Syn is able to bind iron. ITC experiments were focused on iron binding to α -Syn because α -Syn is widely accepted as a copper binding protein. In addition, copper binding to α -Syn had previously been shown in our laboratory. In order to support the proposed function of α -Syn, it was important to show that α -Syn binds iron in the presence of copper. α -Syn was able to bind iron when pre-loaded with copper and with only slightly reduced affinity values. Then ferrozine based experiments were the second step to demonstrate the reduction ability of copper-loaded α -Syn of ferric iron. The core factor of both ITC and ferrozine based assay was the use of freshly purified monomeric α -Syn. There is no clear link between α -Syn structure and its proposed function. ITC showed that α -Syn requires being at monomeric state to bind iron and perform as ferrireductase, which means using the protein after purification before it becomes precipitated or aggregated. Moreover, the ferrozine based assay depends on absorbance measurements, thus any level of aggregation of the protein prior to the experiment would interfere with the out coming results. In addition, such activity of α -Syn might be disturbed by aggregation as it can cause α -Syn to lose its ability to reduce iron. One possibility is that α -Syn aggregation might lead to

changes in the binding sites configuration and therefore losing its ability to bind metals. As a result, metals will accumulate in the brain and become more available for redox activities by producing radicals capable of causing oxidative damage in the brain. On the other hand, conditions which cause copper and iron to increase or accumulate in the brain, have the potential to induce α -Syn aggregation leading to the formation of proteinacious inclusions and iron deposits. Hence, it could be speculated that the presence of α -Syn in a non-aggregated form is important for its activity, especially with the continuous implication of α -Syn aggregation in PD pathogenesis.

It is well known and reported in the literature that metals, such as copper and iron, can induce α -Syn aggregation. This effect was proposed to occur by masking negative charges located at the C-terminus. However, it was essential to demonstrate this effect of metals on WT α -Syn as an initial step in this project using recombinant protein. The second step was then to move on and reveal metals' effect on aggregation of α -Syn mutants which lack the potential metal binding sites. Mutants lacking the potential metal binding sites on α -Syn, were previously created in our laboratory. In order to study the role of metals in protein aggregation it was first necessary to develop a reliable assay to measure α -Syn aggregation. Although ThT assay is considered to be a simple and commonly used method to study protein aggregation, the lack of reproducibility experienced with the assay did not enable the study to go beyond few repeats. Therefore, it was not enough to obtain information about the mechanism of α -Syn aggregation since metals, in the resultant experiments, induced the aggregation of both WT and mutant α -Syn. This assay uses a Thioflavin-T dye to detect the presence of β -sheet rich fibrils. Electron Microscopy (EM) would be a good additional way to confirm the proposed fibril formation.

Looking into aggregation work, and considering there was only one representative experiment of most studied cases it was not enough to draw any conclusions about this work. However, the performed experiments showed the same effect of metals on both WT α -Syn and its metal binding mutants. Metals could be acting by mediating the early stages of aggregation, presumably via metal binding to the protein which is the main core of the project. Particularly, since α -Syn was demonstrated to bind either copper and iron. Nevertheless, deleting amino acids which resemble the metal binding sites did not eliminate the proposed effect on aggregation. Metals can still bind to other sites such as the C-terminus when the N-terminus residues were deleted and thus induce the protein aggregation. The aggregation study was further supported using our cell culture model (SHSY-5Y cells) and western analysis. Copper caused increased formation of high molecular weight aggregates of α -Syn when applied onto SHSY-5Y cells over-expressing WT α -Syn. Formation of such aggregates were present at less degree with cells over-expressing metal binding mutants. However, this is still not enough evidence to propose that induction of aggregation of α -Syn, caused by metals, occur via metal binding to the protein. Each of ThT and western analysis were used for the same goal. They differ in principle but each method identifies a different form of aggregates in response to metal exposure. The former looked into β -sheet rich fibrils, while western blotting provided information about formation of oligomeric aggregates. Therefore, work conducted on assessing α -Syn aggregation can be described as not complete till this point. Further ITC experiments are required for both WT α -Syn and its mutants to clarify the binding mode of metals to these proteins. More importantly is to extend ThT assays with more control over conditions and procedure. The reason why this is important is due to variable factors which were observed to interfere with assay's results. Such factors include possible inaccuracy in protein concentration coming from the method of measurement as well as inconsistencies between protein batches. This is important since protein concentration chosen to be used in the assay could affect protein's structure in solution, therefore the way it folds and aggregates. Also, ThT binding to the β -

sheet structures formed when the protein aggregates is another uncontrollable factor that could vitally influence the assay results. Regarding ThT assay, it is essential to perform further similar experiments using α -Syn mutant in which both N- and C-terminal metal binding sites are deleted. In addition, the use of the cell model as described in aggregation chapter resembles the case where high metal concentrations are present in the brain such as neurodegenerative diseases (Sayre, Moreira et al. 2005). The known threat arising from elevated levels of free copper and iron in PD brains is associated with their ability to promote the formation of reactive oxygen radicals. Such radicals can oxidize proteins and lead to cell death (Sayre, Moreira et al. 2005; Perez and Franz 2010) since oxidized proteins may tend to be insoluble and more prone to aggregate.

My study also presented an attempt to address whether increased metals concentrations would influence the level of α -Syn expression by affecting its promoter activity. However, no new findings were produced in this regard as results varied between no changes to some increase in the promoter activity. Also, our approach was limited to adding metals onto cells and evaluating changes in α -Syn promoter activity using Luciferase assay. Luciferase assay demonstrated a reproducible and a good tool in assessing promoter's activity. It has been reported to be the method of choice in such studies (Chiba-Falek and Nussbaum 2001). It was recently published (McHugh, Wright et al. 2011) that MTF-1, metal transcription factor-1, differentially regulated β -Syn expression and not α -Syn. Such a result links metals with the regulation of synucleins' expression and confirms the importance of a balanced regulation between both α -Syn and β -Syn. β -Syn has been suggested to be a negative regulator of α -Syn (Uversky, Li et al. 2002b), even though it has not been reported to be present in LBs (Biere, Wood et al. 2000). Hence, β -Syn could also represent a therapeutic target in the treatment of synucleinopathies. Furthermore, maintaining metals' levels is crucial for normal brain state and targeting metals, by using metal chelators, is one of the potential approaches in the treatment of PD (Perez and Franz 2010). Mainly

since metals represent a danger when present in increased concentrations due to their ability to oxidise proteins. Copper and iron have been given a central role in PD and other neurodegenerative diseases as well as throughout this thesis. α -Syn binding ability to both metals is essential and might hold other roles besides the one discussed during this work. Sequestering copper by α -Syn may be a part of its natural function as a ferrireductase since α -Syn has no reduction activity in the absence of copper. It is well known that α -Syn has a high affinity to copper (II) at nanomolar range 1:1 stoichiometry (Davies, Wang et al. 2011; Dudzik, Walter et al. 2011). This verifies the use of two equivalents of copper when performing ITC experiments, as it will ensure that copper will bind to α -Syn, yet not very high copper to cause α -Syn aggregation. Moreover, metals such as copper and iron when incubated with α -Syn were shown to form spherical oligomers similar to those formed by α -Syn in the absence of metals (Yamin, Glaser et al. 2003; Lowe, Pountney et al. 2004). Such spherical oligomers were found to be present in α -Syn protofibrils (Ding, Lee et al. 2002). Consequently, this is further evidence that metals have the potential to cause α -Syn transformation from its native state into oligomeric protofibrils, hence make it more prone to aggregate.

Metals were suggested to play a role in the normal brain function since they are involved in CNS metabolism as catalysts, second messengers and gene expression regulators (Minicozzi, Morante et al. 2008). Therefore, metals even at normal levels in the brain may influence α -Syn expression. This is important because it might represent an additional path by which metals induce α -Syn aggregation. Metals could upregulate α -Syn expression; hence increase its accumulation and aggregation. Iron amongst other metals involved in neurodegenerative disorders is known to be the most abundant metal in the human body and brain (Gerlach, Ben-Shachar et al. 1994), and is distributed in an uneven pattern among different brain regions with high levels in the SNpc. Such high content of iron in the brain is essential during development for

functions such as learning and memory. The majority of brain iron is in a bound form and very little is found free in the tissues (Gallagher, Finnegan et al. 2011). However, it represents a hidden danger as injuries to brain cells can release iron ions and cause oxidative stress. Increased iron concentrations can arise from defects in iron metabolism, uptake and storage in the brain (Lieu, Heiskala et al. 2001). Overall, whether metals are related to alterations in α -Syn expression and aggregation, the aggregation process itself can lead to changes in tissue composition in the brain thus altering the normal functional environment. α -Syn aggregation may interrupt its ability to reduce ferric iron (Fe^{3+}) due to possible resultant structural changes of the protein. Therefore, this might suspend ferric iron reduction and increase its level leading to possible oxidative damage and cell death. Such proposal can be proved throughout experimental work covering ferrireductase assay with pre-aggregated α -Syn. The function of other ferrireductases in the brain might also be affected with elevated iron concentrations, while on the other hand any damage to these proteins can cause iron levels to increase. It is ferric iron that increases in such cases. Ferrireductases are important as they enable the reduction of ferric iron into ferrous form that is ready for the cell to use. Iron is delivered to the brain as ferric form mediated by transferrin. Along with aggregated α -Syn, iron deposits were found in Lewy bodies (LBs) (Dexter, Carayon et al. 1991; Hirsch, Brandel et al. 1991; Castellani, Siedlak et al. 2000). Histochemical and biochemical studies have shown an increase in total iron in brains of PD patients in favour of ferric iron (Sofic, Paulus et al. 1991). These findings together prompt thinking that metals are tightly linked to α -Syn role in PD and other neurodegenerative diseases. Transition metals such as copper, iron and zinc were shown to be common dominators in their pathology (Lovell, Robertson et al. 1998; Brown, Guantieri et al. 2004) via interacting with protein targets such as amyloid beta in AD (Selkoe 2001). This interaction was suggested to be mediated by metal-protein association leading to protein aggregation or by metal catalyzed protein oxidation causing protein damage (Bush 2000).

Neurodegenerative diseases represent serious and life-threatening conditions and most of them have no cure. Since such diseases cause neuronal death in the brain, they affect and worsen many of the body's activities such as movement, balance and talking. Clinical symptoms of PD include resting tremor, rigidity and bradykinesia (slowness of movement). With disease progression, other L-dopa responsive motor symptoms evolve such as gait and balance problems, dysarthria and dysphagia (Muller, Wenning et al. 2001; Hely, Morris et al. 2005). With longer disease durations, patients may also develop various non-motor symptoms such as cognitive decline and dementia, depression and psychosis, sleep-wake cycle dysregulation; in addition to pain and sensory symptoms (Poewe and Mahlknecht 2009). Treatment usually focuses on improving symptoms and life quality of the patients. The most commonly used medications are levodopa, dopamine agonists and monoamino oxidase-B inhibitors. In addition, treatment also covers physiotherapy and speech and language therapy. However, treatment targeting the disease symptoms can not cover all needs of late-stage patients. Therefore, slowing disease progression is considered to be a main unachieved requirement in the treatment of PD. Findings in this thesis demonstrated further evidence that interaction between α -Syn and metals has a major implication in PD and cutting off this interaction could represent a potential target into developing therapies. Further research of α -Syn activity as ferrireductase is important. The future work can determine whether this activity could be affected by protein aggregation. In addition, it would be worth to assess the aggregation of α -Syn mutant lacking all potential metal binding sites (at both N- and C-terminals) to evaluate whether observed aggregation is related to metal binding. Capturing images of the end products of aggregation of such mutant will be useful, and then compare them with ones of full length α -Syn. There is currently no precise knowledge about what regulates synucleins, even in disease state. In order to obtain more information about the effect of metals on synuclein expression, it would be useful to use PCR to detect changes in α -Syn levels in cells upon metals addition. It is also beneficial to investigate other

transcription factors related to synuclein expression, since this could represent target for disease therapy.

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